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(54) Title: METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE

(57) Abstract

New methods and reagents for vaccination are described which generate a CD8 T cell immune response against malarial and other antigens such as viral and tumour antigens. Novel vaccination regimes are described which employ a priming composition and a boosting composition, the boosting composition comprising a non-replicating or replication-impaired pox virus vector carrying at least one CD8 T cell epitope which is also present in the priming composition.

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METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE

This invention relates to generation of a protective CD8+ T cell immune response against target antigens using different primer and booster compositions as sources of CD8+ T cell epitopes.

Introduction

A general problem in vaccinology has been an inability to generate high levels of CD8 T cells by immunisation. This has impeded the development of vaccines against several diseases including malaria.

Plasmodium falciparum malaria causes hundreds of millions of malaria infections each year and is responsible for 1-2 million deaths annually. The development of an effective vaccine against malaria is thus a major priority for global public health. A considerable body of immunological research over the last twenty years had led to the identification both of candidate vaccine antigens from the parasite and immunological mechanisms on the host that are likely to protect against infection and disease. However, despite this progress there is still no means of vaccinating against malaria infection which has been shown to be effective in field trials.

A major problem has been the identification of a means of inducing a sufficiently strong immune response in vaccinated individuals to protect against infection and disease. So, although many malaria antigens are known that might be useful in vaccinating against malaria the problem has been how to deliver such antigens or fragments of them known as epitopes, which are recognised by cells of the immune system, in a way that induces a sufficiently strong immune response of a particular type.

It has been known for many years that it is possible to protect individuals by immunising them with very large doses of irradiated malaria sporozoites given by bites from infected mosquitoes. Although this is a

wholly impractical method of mass vaccination it has provided a model for analysing the immune responses that might be mediating protective immunity against sporozoite infection (Nardin and Nussenzweig 1993).

A considerable amount of research over the last decade or more has indicated that a major protective immune response against the early pre-erythrocytic stage of *P. falciparum* malaria is mediated by T lymphocytes of the CD8+ ve type (CD8+ T cells). Such cells have been shown to mediate protection directly in mouse models of malaria infection (Nardin and Nussenzweig 1993). Such T cells have also been identified in individuals naturally exposed to malaria and in volunteers immunised with irradiated sporozoites (Hill *et al.* 1991; Aidoo *et al.* 1995; Wizel *et al.* 1995). There is much indirect evidence that such CD8+ T cells are protective against malaria infection and disease in humans (Lalvani *et al.* 1994).

CD8+ T cells may function in more than one way. The best known function is the killing or lysis of target cells bearing peptide antigen in the context of an MHC class I molecule. Hence these cells are often termed cytotoxic T lymphocytes (CTL). However, another function, perhaps of greater protective relevance in malaria infections is the ability of CD8+ T cells to secrete interferon gamma (IFN-γ). Thus assays of lytic activity and of IFN-γ release are both of value in measuring a CD8+ T cell immune response. In malaria these CD8+ve cells can protect by killing the parasite at the early intrahepatic stage of malaria infection before any symptoms of disease are produced (Seguin *et al.* 1994) .

The agent of fatal human malaria, *P. falciparum* infects a restricted number of host species: humans, chimpanzees and some species of New World monkey. The best non-human model of malaria is the chimpanzee because this species is closely related to humans and liver-stage infection is observed consistently unlike in the monkey hosts (Thomas *et al.* 1994). Because of the expense and limited availability of chimpanzees most laboratory studies of malaria are performed in mice,

using the rodent malaria species *P. berghei* or *P. yoelii*. These latter two models are well studied and it has been shown in both that CD8+ve lymphocytes play a key role in protective immunity against sporozoite challenge.

Previous studies have assessed a large variety of means of inducing CD8+ T cell responses against malaria. Several of these have shown some level of CD8+ T cell response and partial protection against malaria infection in the rodent models (e.g. Li *et al.* 1993; Sedegah *et al* 1994; Lanar *et al.* 1996). However, an effective means of immunising with subunit vaccines by the induction of sufficiently high levels of CD8+ T lymphocytes to protect effectively against malaria sporozoite infection has not previously been demonstrated.

In recent years improved immune responses generated to potential vaccines have been sought by varying the vectors used to deliver the antigen. There is evidence that in some instances antibody responses are improved by using two different vectors administered sequentially as prime and boost. A variety of combinations of prime and boost have been tested in different potential vaccine regimes.

Leong *et al.* (Vaccines 1995, 327-331) describe immunising mice firstly to DNA expressing the influenza haemagglutinin (HA) antigen and then with a recombinant fowlpox vector expressing HA. An enhanced antibody response was obtained following boosting.

Richmond *et al.* (Virology 1997, 230: 265-274) describe attempts to raise neutralising antibodies against HIV-1 env using DNA priming and recombinant vaccinia virus boosting. Only low levels of antibody responses were observed with this prime boost regime and the results were considered disappointing.

Fuller *et al.* (Vaccine 1997, 15:924-926 and Immunol Cell Biol 1997, 75:389-396) describe an enhancement of antibody responses to DNA immunisation of macaques by using a booster immunisation with

replicating recombinant vaccinia viruses. However, this did not translate into enhanced protective efficacy as a greater reduction in viral burden and attenuation of CD4 T cell loss was seen in the DNA primed and boosted animals.

Hodge *et al* (Vaccine 1997, 15: 759-768) describe the induction of lymphoproliferative T cell responses in a mouse model for cancer using human carcinoembryonic antigen (CEA) expressed in a recombinant fowl pox virus (ALVAC). The authors primed an immune response with CEA-recombinant replication competent vaccinia viruses of the Wyeth or WR strain and boosted the response with CEA-recombinant ALVAC. This led to an increase in T cell proliferation but did not result in enhanced protective efficacy if compared to three wild type recombinant immunisations (100% protection), three recombinant ALVAC-CEA immunisations (70% protection) or WR prime followed by two ALVAC-CEA immunisations (63% protection).

Thus some studies of heterologous prime-boost combination have found some enhancement of antibody and lymphoproliferative responses but no significant effect on protective efficacy in an animal model. CD8 T cells were not measured in these studies. The limited enhancement of antibody response probably simply reflects the fact that antibodies to the priming immunogen will often reduce the immunogenicity of a second immunisation with the same immunogen, while boosting with a different carrier will in part overcome this problem. This mechanism would not be expected to be significantly affected by the order of immunisation.

Evidence that a heterologous prime boost immunisation regime might affect CD8 T cell responses was provided by Li *et al.* (1993). They described partial protective efficacy induced in mice against malaria sporozoite challenge by administering two live viral vectors, a recombinant replicating influenza virus followed by a recombinant replicating vaccinia virus encoding a malaria epitope. Reversing the order of immunisation led

to loss of all protective efficacy and the authors suggested that this might be related to infection of liver cells by vaccinia, resulting in localisation of CTLs in the liver to protect against the hepatocytic stages of malaria parasites.

Rodrigues *et al.* (J. Immunol. 1994, 4636-4648) describe immunising mice with repeated doses of a recombinant influenza virus expressing an immunodominant B cell epitope of the malarial circumsporozoite (CS) protein followed by a recombinant vaccinia virus booster. The use of a wild type vaccinia strain and an attenuated but replication-competent vaccinia strain in the booster yielded very similar levels of partial protection. However the attenuated but replication competent strain was slightly less immunogenic for priming CD8 T cells than the wild type vaccinia strain.

Murata *et al.* (Cell. Immunol. 1996, 173: 96-107) reported enhanced CD8 T cell responses after priming with replicating recombinant influenza viruses and boosting with a replicating strain of vaccinia virus and suggested that the partial protection observed in the two earlier studies was attributable to this enhanced CD8 T cell induction.

Thus these three studies together provide evidence that a booster immunisation with a replicating recombinant vaccinia virus may enhance to some degree CD8 T cell induction following priming with a replicating recombinant influenza virus. However, there are two limitations to these findings in terms of their potential usefulness. Firstly, the immunogenicity induced was only sufficient to achieve partial protection against malaria and even this was dependent on a highly immunogenic priming immunisation with an unusual replicating recombinant influenza virus. Secondly, because of the potential and documented side-effects of using these replicating viruses as immunogens these recombinant vectors are not suitable for general human use as vaccines.

Modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. MVA was derived by serial passage >500 times in chick embryo fibroblasts (CEF) of material derived from a pox lesion on a horse in Ankara, Turkey (Mayr *et al.* 1975). It was shown to be replication-impaired yet able to induce protective immunity against veterinary poxvirus infections (Mayr 1976). MVA was used as a human vaccine in the final stages of the smallpox eradication campaign, being administered by intracutaneous, subcutaneous and intramuscular routes to >120,000 subjects in southern Germany. No significant side effects were recorded, despite the deliberate targeting of vaccination to high risk groups such as those with eczema (Mayr *et al.* 1978; Stickl *et al.* 1974; Mahnel *et al.* 1994;). The safety of MVA reflects the avirulence of the virus in animal models, including irradiated mice and following intracranial administration to neonatal mice. The non-replication of MVA has been correlated with the production of proliferative white plaques on chick chorioallantoic membrane, abortive infection of non-avian cells, and the presence of six genomic deletions totalling approximately 30 kb (Meyer *et al.* 1991). The avirulence of MVA has been ascribed partially to deletions affecting host range genes K1L and C7L, although limited viral replication still occurs on human TK-143 cells and African Green Monkey CV-1 cells (Altenburger *et al.* 1989). Restoration of the K1L gene only partially restores MVA host range (Sutter *et al.* 1994). The host range restriction appears to occur during viral particle maturation, with only immature virions being observed in human HeLa cells on electron microscopy (Sutter *et al.* 1992). The late block in viral replication does not prevent efficient expression of recombinant genes in MVA. Recombinant MVA expressing influenza nucleoprotein, influenza haemagglutinin, and SIV proteins have proved to be immunogenic and provide varying degrees of protection in animal models, although this has never been ascribed to CD8+ T lymphocytes

alone (Sutter *et al.* 1994, Hirsch *et al.* 1995; Hirsch *et al.* 1996). Recombinant MVA is considered a promising human vaccine candidate because of these properties of safety and immunogenicity (Moss *et al.* 1995). Recombinant MVA containing DNA which codes for foreign antigens is described in US 5,185,146 (Altenburger).

Poxviruses have evolved strategies for evasion of the host immune response that include the production of secreted proteins that function as soluble receptors for tumour necrosis factor, IL-1β, interferon (IFN)-α/β and IFN-γ, which normally have sequence similarity to the extracellular domain of cellular cytokine receptors (Symons *et al.* 1995; Alcami *et al.* 1995; Alcami *et al.* 1992). The most recently described receptor of this nature is a chemokine receptor (Graham *et al.* 1997). These viral receptors generally inhibit or subvert an appropriate host immune response, and their presence is associated with increased pathogenicity. The Il-1β receptor is an exception: its presence diminishes the host febrile response and enhances host survival in the face of infection (Alcami *et al.* 1996). We have discovered that MVA lacks functional cytokine receptors for interferon γ, interferon αβ, Tumour Necrosis Factor and CC chemokines, but it does possess the potentially beneficial IL-1β receptor. MVA is the only known strain of vaccinia to possess this cytokine receptor profile, which theoretically renders it safer and more immunogenic than other poxviruses. Another replication-impaired and safe strain of vaccinia known as NYVAC is fully described in Tartaglia *et al.*(Virology 1992, 188: 217-232).

It has long been recognised that live viruses have some attractive features as recombinant vaccine vectors including a high capacity for foreign antigens and fairly good immunogenicity for cellular immune responses (Ellis 1988 new technologies for making vaccines. In: Vaccines. Editors: Plotkin S A and Mortimer E A. W B Saunders, Philadelphia, page 568; Woodrow G C 1977. In: New Generation

Vacciness 2nd Edition. Editors: Levine M M, Woodrow G C, Kaper J B, Cobon G, page 33). This has led to attempts to attenuate the virulence of such live vectors in various ways including reducing their replication capacity (Tartaglia J *et al.* 1992 Virology 188: 217-232). However such a reduction in replication reduces the amount of antigen produced by the virus and thereby would be expected to reduce vaccine immunogenicity. Indeed attenuation of replicating vaccinia strains has previously been shown to lead to some substantial reductions in antibody responses (Lee M S *et al*, 1992 J Virology 66: 2617-2630). Similarly the non-replicating fowlpox vector was found to be less immunogenic for antibody production and less protective than a replicating wild-type vaccinia strain in a rabies study (Taylor J *et al.* 1991 Vaccine 9: 190-193).

It has now been discovered that non-replicating and replication-impaired strains of poxvirus provide vectors which give an extremely good boosting effect to a primed CTL response. Remarkably, this effect is significantly stronger than a boosting effect by wild type poxviruses. The effect is observed with malarial and other antigens such as viral and tumour antigens, and is protective as shown in mice and non-human primate challenge experiments. Complete rather than partial protection from sporozoite challenge has been observed with the novel immunisation regime.

It is an aim of this invention to identify an effective means of immunising against malaria. It is a further aim of this invention to identify means of immunising against other diseases in which CD8+ T cell responses play a protective role. Such diseases include but are not limited to infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and

Trypanosoma; and certain forms of cancer e.g. melanoma, cancer of the breast and cancer of the colon.

We describe here a novel method of immunising that generated very high levels of CD8+ T cells and was found to be capable of inducing unprecedented complete protection against *P. berghei* sporozoite challenge. The same approach was tested in higher primates and found to be highly immunogenic in this species also, and was found to induce partial protection against *P. falciparum* challenge. Induction of protective immune responses has also been demonstrated in two additional mouse models of viral infection and cancer.

We show further than the novel immunisation regime that is described here is also effective in generating strong CD8+ T cell responses against HIV epitopes. Considerable evidence indicates that the generation of such CD8+ T cell responses can be expected to be of value in prophylactic or therapeutic immunisation against this viral infection and disease (Gallimore *et al* 1995; Ada 1996). We demonstrate that strong CD8+T cell responses may be generated against epitopes from both HIV and malaria using an epitope string with sequences from both of these micro-organisms. The success in generating enhanced immunogenicity against both HIV and malaria epitopes, and also against influenza and tumour epitopes, indicates that this novel immunisation regime can be effective generally against many infectious pathogens and also in non-infectious diseases where the generation of a strong CD8+ T cell response may be of value.

A surprising feature of the current invention is the finding of the very high efficacy of non-replicating agents in both priming and particularly in boosting a CD8+ T cell response. In general the immunogenicity of CD8+ T cell induction by live replicating viral vectors has previously been found to be higher than for non-replicating agents or replication-impaired vectors. This is as would be expected from the greater

amount of antigen produced by agents that can replicate in the host. Here however we find that the greatest immunogenicity and protective efficacy is surprisingly observed with non-replicating vectors. The latter have an added advantage for vaccination in that they are in general safer for use in humans than replicating vectors.

The present invention provides in one aspect a kit for generating a protective CD8+ T cell immune response against at least one target antigen, which kit comprises:

(i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and

(ii) a boosting composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;

with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.

In another aspect the invention provides a method for generating a protective CD8+ T cell immune response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to the invention.

Preferably, the source of CD8+ T cell epitopes in (i) in the method according to the invention is a non-viral vector or a non-replicating or replication-impaired viral vector, although replicating viral vectors may be used.

Preferably, the source of CD8+ T cell epitopes in (i) is not a poxvirus vector, so that there is minimal cross-reactivity between the primer and the booster.

In one preferred embodiment of the invention, the source of CD8+ T cell epitopes in the priming composition is a nucleic acid, which may be DNA or RNA, in particular a recombinant DNA plasmid. The DNA or RNA may be packaged, for example in a lysosome, or it may be in free form.

In another preferred embodiment of the invention, the source of CD8+ T cell epitopes in the priming composition is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen. Polyproteins include two or more proteins which may be the same, or preferably different, linked together. Particularly preferred in this embodiment is a recombinant proteinaceous particle such as a Ty virus-like particle (VLP) (Burns *et al.* Molec. Biotechnol. 1994, 1: 137-145).

Preferably, the source of CD8+ T cell epitopes in the boosting composition is a vaccinia virus vector such as MVA or NYVAC. Most preferred is the vaccinia strain modified virus ankara (MVA) or a strain derived therefrom. Alternatives to vaccinia vectors include avipox vectors such as fowlpox or canarypox vectors. Particularly suitable as an avipox vector is a strain of canarypox known as ALVAC (commercially available as Kanapox), and strains derived therefrom.

Poxvirus genomes can carry a large amount of heterologous genetic information. Other requirements for viral vectors for use in vaccines include good immunogenicity and safety. MVA is a replication-impaired vaccinia strain with a good safety record. In most cell types and normal human tissues, MVA does not replicate; limited replication of MVA is observed in a few transformed cell types such as BHK21 cells. It has now been shown, by the results described herein, that recombinant MVA

and other non-replicating or replication-impaired strains are surprisingly and significantly better than conventional recombinant vaccinia vectors at generating a protective CD8+ T cell response, when administered in a boosting composition following priming with a DNA plasmid, a recombinant Ty-VLP or a recombinant adenovirus.

It will be evident that vaccinia virus strains derived from MVA, or independently developed strains having the features of MVA which make MVA particularly suitable for use in a vaccine, will also be suitable for use in the invention.

MVA containing an inserted string of epitopes (MVA-HM, which is described in the Examples) has been deposited at the European Collection of Animal Cell Cultures, CAMR, Salisbury, Wiltshire SP4 0JG, UK under accession no. V97060511 on 5 June 1997.

The term "non-replicating" or "replication-impaired" as used herein means not capable of replication to any significant extent in the majority of normal mammalian cells or normal human cells. Viruses which are non-replicating or replication-impaired may have become so naturally (i.e. they may be isolated as such from nature) or artificially e.g. by breeding *in vitro* or by genetic manipulation, for example deletion of a gene which is critical for replication. There will generally be one or a few cell types in which the viruses can be grown, such as CEF cells for MVA.

Replication of a virus is generally measured in two ways: 1) DNA synthesis and 2) viral titre. More precisely, the term "non-replicating or replication-impaired" as used herein and as it applies to poxviruses means viruses which satisfy either or both of the following criteria:

1) exhibit a 1 log (10 fold) reduction in DNA synthesis compared to the Copenhagen strain of vaccinia virus in MRC-5 cells (a human cell line);

2) exhibit a 2 log reduction in viral titre in HELA cells (a human cell line) compared to the Copenhagen strain of vaccinia virus.

Examples of poxviruses which fall within this definition are MVA, NYVAC and avipox viruses, while a virus which falls outside the definition is the attenuated vaccinia strain M7.

Alternative preferred viral vectors for use in the priming composition according to the invention include a variety of different viruses, genetically disabled so as to be non-replicating or replication-impaired. Such viruses include for example non-replicating adenoviruses such as E1 deletion mutants. Genetic disabling of viruses to produce non-replicating or replication-impaired vectors has been widely described in the literature (e.g. McLean *et al.* 1994).

Other suitable viral vectors for use in the priming composition are vectors based on herpes virus and Venezuelan equine encephalitis virus (VEE) (Davies *et al.* 1996). Suitable bacterial vectors for priming include recombinant BCG and recombinant *Salmonella* and *Salmonella* transformed with plasmid DNA (Darji A *et al.* 1997 Cell 91: 765-775).

Alternative suitable non-viral vectors for use in the priming composition include lipid-tailed peptides known as lipopeptides, peptides fused to carrier proteins such as KLH either as fusion proteins or by chemical linkage, whole antigens with adjuvant, and other similar systems. Adjuvants such as QS21 or SBAS2 (Stoute J A *et al.* 1997 N Engl J Medicine 226: 86-91) may be used with proteins, peptides or nucleic acids to enhance the induction of T cell responses. These systems are sometimes referred to as "immunogens" rather than "vectors", but they are vectors herein in the sense that they carry the relevant CD8+ T cell epitopes.

There is no reason why the priming and boosting compositions should not be identical in that they may both contain the

priming source of CD8+ T cell epitopes as defined in (i) above and the boosting source of CD8+ T cell epitopes as defined in (ii) above. A single formulation which can be used as a primer and as a booster will simplify administration. The important thing is that the primer contains at least the priming source of epitopes as defined in (i) above and the booster contains at least the boosting source of epitopes as defined in (ii) above.

The CD8+ T cell epitopes either present in, or encoded by the priming and boosting compositions, may be provided in a variety of different forms, such as a recombinant string of one or two or more epitopes, or in the context of the native target antigen, or a combination of both of these. CD8+ T cell epitopes have been identified and can be found in the literature, for many different diseases. It is possible to design epitope strings to generate a CD8+ T cell response against any chosen antigen that contains such epitopes. Advantageously, the epitopes in a string of multiple epitopes are linked together without intervening sequences so that unnecessary nucleic acid and/or amino acid material is avoided. In addition to the CD8+ T cell epitopes, it may be preferable to include one or more epitopes recognised by T helper cells, to augment the immune response generated by the epitope string. Particularly suitable T helper cell epitopes are ones which are active in individuals of different HLA types, for example T helper epitopes from tetanus (against which most individuals will already be primed). A useful combination of three T helper epitopes is employed in the examples described herein. It may also be useful to include B cell epitopes for stimulating B cell responses and antibody production.

The priming and boosting compositions described may advantageously comprise an adjuvant. In particular, a priming composition comprising a DNA plasmid vector may also comprise granulocyte macrophage-colony stimulating factor (GM-CSF), or a plasmid

encoding it, to act as an adjuvant; beneficial effects are seen using GM-CSF in polypeptide form.

The compositions described herein may be employed as therapeutic or prophylactic vaccines. Whether prophylactic or therapeutic immunisation is the more appropriate will usually depend upon the nature of the disease. For example, it is anticipated that cancer will be immunised against therapeutically rather than before it has been diagnosed, while anti-malaria vaccines will preferably, though not necessarily be used as a prophylactic.

The compositions according to the invention may be administered via a variety of different routes. Certain routes may be favoured for certain compositions, as resulting in the generation of a more effective response, or as being less likely to induce side effects, or as being easier for administration. The present invention has been shown to be effective with gene gun delivery, either on gold beads or as a powder.

In further aspects, the invention provides:

- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a non-replicating or replication-impaired recombinant pox virus encoding the same epitope or antigen;
- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen;

- the use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response;
- the use of an MVA vector in the manufacture of a medicament for boosting a CD8+ T cell immune response;
- a medicament for boosting a primed CD8+ T cell response against at least one target antigen or epitope, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier; and
- the priming and/or boosting compositions described herein, in particulate form suitable for delivery by a gene gun; and methods of immunisation comprising delivering the compositions by means of a gene gun.

Also provided by the invention are: the epitope strings described herein, including epitope strings comprising the amino acid sequences listed in table 1 and table 2; recombinant DNA plasmids encoding the epitope strings; recombinant Ty-VLPs comprising the epitope strings; a recombinant DNA plasmid or non-replicating or replication impaired recombinant pox virus encoding the *P. falciparum* antigen TRAP; and a recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes in sequence such as CTL epitopes from malaria.

Example Formulations and Immunisation Protocols

Formulation 1

Priming Composition: DNA plasmid 1 mg/ml in PBS

Boosting Composition: Recombinant MVA, 10^8 ffu in PBS

Protocol: Administer two doses of 1 mg of priming composition, i.m., at 0 and 3 weeks followed by two doses of booster intradermally at 6 and 9 weeks.

Formulation 2

Priming Composition: Ty-VLP 500μg in PBS

Boosting Composition: MVA, 10^8 ffu in PBS

Protocol: Administer two doses of priming composition, i.m., at 0 and 3 weeks, then 2 doses of booster at 6 and 9 weeks. For tumour treatment, MVA is given i.v. as one of most effective routes.

Formulation 3

Priming Composition: Protein 500μg + adjuvant (QS-21)

Boosting Composition: Recombinant MVA, 10^8 ffu in PBS

Protocol: Administer two doses of priming composition at 0 and 3 weeks and 2 doses of booster i.d. at 6 and 9 weeks.

Formulation 4

Priming Composition: Adenovirus vector, 10^9 pfu in PBS

Boosting Composition: Recombinant MVA, 10^8 ffu in PBS

Protocol: Administer one or two doses of priming composition intradermally at 0 and 3 weeks and two doses of booster i.d. at 6 and 9 weeks.

The above doses and protocols may be varied to optimise protection. Doses may be given between for example, 1 to 8 weeks apart rather than 2 weeks apart.

The invention will now be further described in the examples which follow.

EXAMPLES

EXAMPLE 1

Materials and Methods

Generation of the epitope strings.

The malaria epitope string was made up of a series of cassettes each encoding three epitopes as shown in Table 1, with restriction enzyme sites at each end of the cassette. Each cassette was constructed from four synthetic oligonucleotides which were annealed together, ligated into a cloning vector and then sequenced to check that no errors had been introduced. Individual cassettes were then joined together as required. The Bam*HI* site at the 3' end of cassette C was fused to the Bgl*II* site at the 5' end of cassette A, destroying both restriction enzyme sites and encoding a two amino acid spacer (GS) between the two cassettes. Cassettes B, D and H were then joined to the string in the same manner. A longer string containing CABDHFE was also constructed in the same way.

Table 1. CTL epitopes of the malaria (M) string

Cassette	Epitope	Amino acid Sequence	DNA sequence	Type	HLA restriction
A	Ls8	KPNDKSLY	AAGCCGAACGACAAGTCCTTGTAT	CTL	B35
	Cp26	KPKDELDY	AAACCTAAGGACGAATTGGACTAC	CTL	B35
	Ls6	KPIVQYDNF	AAGCCAATCGTTCAATACGACAACTTC	CTL	B53
B	Tr42/43	ASKNKEKALII	GCCCCAAGAACAAGGAAAAGGCTTTGATCATC	CTL	B8
	Tr39	GIAGGLALL	GGTATCGCTGGTGGTTTGGCCTTGTTG	CTL	A2.1
	Cp6	MNPNDPNRNV	ATGAACCCTAATGACCCAAACAGAAACGTC	CTL	B7

C	St8	MINAYLDKL	ATGATCAACGCCTACTTGGACAAGTTG	CTL	A2.2
	Ls50	ISKYEDEI	ATCTCCAAGTACGAAGACGAAATC	CTL	B17
	Pb9	SYIPSAEKI	TCCTACATCCCATCTGCCGAAAAGATC	CTL	mouse H2-K^d
D	Tr26	HLGNVKYLV	CACTTGGGTAACGTTAAGTACTTGGTT	CTL	A2.1
	Ls53	KSLYDEHI	AAGTCTTTGTACGATGAACACATC	CTL	B58
	Tr29	LLMDCSGSI	TTATTGATGGACTGTTCTGGTTCTATT	CTL	A2.2
E	NANP	NANPNANPN ANPNANP	AACGCTAATCCAAACGCAAATCCGAACGCCA ATCCTAACGCGAATCCC	B cell	
	TRAP AM	DEWSPCSVT CGKGTRSRK RE	GACGAATGGTCTCCATGTTCTGTCACTTGTG GTAAGGGTACTCGCTCTAGAAAGAGAGAA	Heparin binding motif	
F	Cp39	YLNKIQNSL	TACTTGAACAAAATTCAAAACTCTTTG	CTL	A2.1
	La72	MEKLKELEK	ATGGAAAAGTTGAAAGAATTGGAAAAG	CTL	B8
	ex23	ATSVLAGL	GCTACTTCTGTCTTGGCTGGTTTG	CTL	B58
H	CSP	DPNANPNVD PNANPNV	GACCCAAACGCTAACCCAAACGTTGACCCA AACGCCAACCCAAACGTC	T helper	Universal
	BCG	QVHFQPLPP AVVKL	CAAGTTCACTTCCAACCATTGCCTCCGGCCG TTGTCAAGTTG	T helper	epitopes
	TT	QFIKANSKFI GITE	CAATTCATCAAGGCCAACTCTAAGTTCATCG GTATCACCGAA	T helper	

Table 1 Sequences included in the malaria epitope string. Each cassette consists of the epitopes shown above, in the order shown, with no additional sequence between epitopes within a cassette. A Bgl*II* site was added at the 5' end and a Bam*HI* site at the 3' end, such that between cassettes in an epitope string the Bam*HI*/Bgl*II* junction encodes GS. All epitopes are from *P. falciparum* antigens except for pb9 *(P. berghei),* BCG *(M. tuberculosis)* and TT (Tetanus). The amino acid and DNA sequences shown in the table have SEQ ID NOS. 1 to 40 in the order in which they appear.

Figure 1 shows the construct used to express Ty-VLP with the malaria epitope cassette CABDHFE. CTL epitopes are from *P. falciparum* STARP (sporozoite threonine- and asparagine-rich protein) (st), LSA-1 (liver stage antigen 1) (1s), CSP (circumsporozoite protein) (cp),

TRAP (thrombospondin-related adhesive protein) (tr), LSA-3 (liver stage antigen 3) (la) and Exp-1 (exported protein 1) (ex). Helper epitopes are from the *P. falciparum* CS protein, the *M. tuberculosis* 38Kd antigen and Tetanus Toxoid. NANP is the antibody epitope from CS and AM is the adhesion motif from *P. falciparum* TRAP (Muller *et al* 1993). The length of the complete string is 229 amino acids as shown in the table 1 legend, with the amino acid sequence:-

MINAYLDKLISKYEDEISYIPSAEKIGSKPNDKSLYKPKDELDYKPIVQYDN
FGSASKNKEKALIIGIAGGLALLMNPNDPNRNVGSHLGNVKYLVKSLYDE
HILLMDCSGSIGSDPNANPNVDPNANPNVQVHFQPLPPAVVKLQFIKANS
KFIGITEGSYLNKIQNSLMEKLKELEKATSVLAGLGSNANPNANPNANPNA
NPDEWSPCSVTCGKGTRSRKREGSGK [SEQ ID NO: 41].

The HIV epitope string was also synthesised by annealing oligonucleotides. Finally the HIV and malaria epitope strings were fused by joining the *Bam*HI site at the 3' end of the HIV epitopes to the *Bgl*II site at the 5' end of cassettes CAB to form the HM string (Table 2)

Table 2 CTL epitopes of the HIV/SIV epitope string

Epitope	Restriction	Origin
YLKDQQLL	A24, B8	HIV-1 gp41
ERYLKDQQL	B14	HIV-1 gp41
EITPIGLAP	Mamu-B*01	SIV env
PPIPVGEIY	B35	HIV-1 p24
GEIYKRWII	B8	HIV-1 p24
KRWIILGLNK	B*2705	HIV-1 p24
IILGLNKIVR	A33	HIV-1 p24
LGLNKIVRMY	Bw62	HIV-1 p24
YNLTMKCR	Mamu-A*02	SIV env
RGPGRAFVTI	A2, H-2Dd	HIV-1 gp120
GRAFVTIGK	B*2705	HIV-1 gp120
TPYDINQML	B53	HIV-2 gag
CTPYDINQM	Mamu-A*01	SIV gag

RPQVPLRPMTY	B51	HIV-1 nef
QVPLRPMTYK	A*0301, A11	HIV-1 nef
VPLRPMTY	B35	HIV-1 nef
AVDLSHFLK	A11	HIV-1 nef
DLSHFLKEK	A*0301	HIV-1 nef
FLKEKGGL	B8	HIV-1 nef
ILKEPVHGV	A*0201	HIV-1 pol
ILKEPVHGVY	Bw62	HIV-1 pol
HPDIVIYQY	B35	HIV-1 pol
VIYQYMDDL	A*0201	HIV-1 pol

Table 2 Sequences of epitopes from HIV or SIV contained in the H epitope string and assembled as shown in figure 2. The amino acids in the table have SEQ ID NOS: 42 to 64 in the order in which they appear.

Figure 2 shows a schematic outline of the H, M and HM proteins. The bar patterns on the schematic representations of the polyepitope proteins indicate the origin of the sequences (see tables 1 and 2). The positions of individual epitopes and their MHC restrictions are depicted above and below the proteins. Pb is the only epitope derived from the protein of *P. berghei*. All other epitopes in the M protein originate from proteins of *P. falciparum*: cs – circumsporozoite protein, st – STARP, ls – LSA-1 and tr – TRAP. BCG – 38 kDa protein of *M. tuberculosis*; TT – tetanus toxin.

For the anti-tumour vaccine an epitope string containing CTL epitopes was generated, similar to the malaria and HIV epitope string. In this tumour epitope string published murine CTL epitopes were fused together to create the tumour epitope string with the amino acid sequence: **MLPYLGWLVF-AQHPNAELL-KHYLFRNL-SPSYVYHQF-IPNPLLGLD** [SEQ ID NO: 65]. CTL epitopes shown here were fused together. The first amino acid methionine was introduced to initiate translation.

Ty virus-like particles (VLPs).

The epitope string containing cassette CABDH was introduced into a yeast expression vector to make a C-terminal in-frame fusion to the TyA protein. When TyA or TyA fusion proteins are expressed in yeast from this vector, the protein spontaneously forms virus like particles which can be purified from the cytoplasm of the yeast by sucrose gradient centrifugation. Recombinant Ty-VLPs were prepared in this manner and dialysed against PBS to remove the sucrose before injection (c.f. Layton *et al.* 1996).

Adenoviruses

Replication-defective recombinant Adenovirus with a deletion of the E1 genes was used in this study (McGrory et al, 1988). The Adenovirus expressed *E. coli* β-galactosidase under the control of a CMV IE promoter. For immunisations, 10^7 pfu of virus were administered intradermally into the ear lobe.

Peptides

Peptides were purchased from Research Genetics (USA), dissolved at 10 mg/ml in DMSO (Sigma) and further diluted in PBS to 1 mg/ml. Peptides comprising CTL epitopes that were used in the experiments described herein are listed in table 3

Table 3 Sequence of CTL peptide epitopes

sequence	Antigen	MHC restriction
LPYLGWLVF	P1A tumour antigen	L^d
SYIPSAEKI	*P. berghei* CSP	K^d
RGPGRAFVTI	HIV gag	D^d

TPHPARIGL	*E. coli* β-galactosidase	L^d
TYQRTRALV	Influenza A virus NP	K^d
SDYEGRLI	Influenza A virus NP	K^k
ASNENMETM	Influenza A virus NP	D^b
INVAFNRFL	*P. falciparum* TRAP	K^b

The amino acid sequences in Table 3 have SEQ ID NOS: 66 to 73, in the order in which they appear in the Table.

Plasmid DNA constructs

A number of different vectors were used for constructing DNA vaccines. Plasmid pTH contains the CMV IE promoter with intron A, followed by a polylinker to allow the introduction of antigen coding sequences and the bovine growth hormone transcription termination sequence. The plasmid carries the ampicillin resistance gene and is capable of replication in *E. coli* but not mammalian cells. This was used to make DNA vaccines expressing each of the following antigens: *P. berghei* TRAP, *P. berghei* CS, *P. falciparum* TRAP, *P. falciparum* LSA-1 (278 amino acids of the C terminus only), the epitope string containing cassettes CABDH and the HM epitope string (HIV epitopes followed by cassettes CAB). Plasmid pSG2 is similar to pTH except for the antibiotic resistance gene. In pSG2 the ampicillin resistance gene of pTH has been replaced by a kanamycin resistance gene. pSG2 was used to to make DNA vaccines expressing the following antigens: P. berghei PbCSP, a mouse tumour epitope string, the epitope string containing cassettes CABDH and the HM epitope string. Plasmid V1J-NP expresses influenza nucleoprotein under the control of a CMV IE promoter. Plasmids CMV-TRAP and CMV-LSA-1 are similar to pTH.TRAP and pTH. LSA-1 but do not contain intron A of the CMV promoter. Plasmids RSV.TRAP and RSV.LSA-1 contain the RSV promoter, SV40 transcription termination sequence and are tetracycline

resistant. For induction of β-galactosidase-specific CTL plasmid pcDNA3/His/LacZ (Invitrogen) was used. All DNA vaccines were prepared from *E. coli* strain DH5α using Qiagen plasmid purification columns.

Generation of recombinant vaccinia viruses

Recombinant MVAs were made by first cloning the antigen sequence into a shuttle vector with a viral promoter such as the plasmid pSC11 (Chakrabarti *et al.* 1985; Morrison *et al.* 1989). *P. berghei* CS and *P. falciparum* TRAP, influenza nucleoprotein and the HM and mouse tumour epitope polyepitope string were each expressed using the P7.5 promoter (Mackett *et al.* 1984), and *P. berghei* TRAP was expressed using the strong synthetic promoter (SSP; Carroll *et al.* 1995). The shuttle vectors, pSC11 or pMCO3 were then used to transform cells infected with wild-type MVA so that viral sequences flanking the promoter, antigen coding sequence and marker gene could recombine with the MVA and produce recombinants. Recombinant viruses express the marker gene (β glucuronidase or β galactosidase) allowing identification of plaques containing recombinant virus. Recombinants were repeatedly plaque purified before use in immunisations. The recombinant NYVAC-PbCSP vaccinia was previously described (Lanar *et al.* 1996). The wild type or Western Reserve (WR) strain of recombinant vaccinia encoding PbCSP was described previously (Satchidanandam *et al.* 1991).

Cells and culture medium

Murine cells and Epstein-Barr virus transformed chimpanzee and macaque B cells (BCL) were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS). Splenocytes were restimulated with the peptides indicated (final concentration 1 μg/ml) in MEM medium with 10% FCS, 2mM glutamine, 50U/ml penicillin, 50 μM 2-mercaptoethanol and 10mM Hepes pH7.2 (Gibco, UK).

Animals

Mice of the strains indicated, 6-8 weeks old were purchased from Harlan Olac (Shaws Farm, Blackthorn, UK). Chimpanzees H1 and H2 were studied at the Biomedical Primate Research Centre at Rijswick, The Netherlands. Macaques were studied at the University of Oxford.

Immunisations

Plasmid DNA immunisations of mice were performed by intramuscular immunisation of the DNA into the musculus tibialis under anaesthesia. Mouse muscle was sometimes pre-treated with 50 µl of 1mM cardiotoxin (Latoxan, France) 5-9 days prior to immunisation as described by Davis *et al* (1993), but the presence or absence of such pre-treatment was not found to have any significant effect on immunogenicity or protective efficacy. MVA immunisation of mice was performed by either intramuscular (i.m.), intravenous (into the lateral tail vein) (i.v.), intradermal (i.d.), intraperitoneal (i.p.) or subcutaneous (s.c.) immunisation. Plasmid DNA and MVA immunisation of the chimpanzees H1 and H2 was performed under anaesthesia by intramuscular immunisation of leg muscles. For these chimpanzee immunisations the plasmid DNA was co-administered with 15 micrograms of human GM-CSF as an adjuvant. Recombinant MVA administration to the chimpanzees was by intramuscular immunisation under veterinary supervision. Recombinant human GM-CSF was purchased from Sandoz (Camberley, UK). For plasmid DNA immunisations using a gene gun, DNA was precipitated onto gold particles. For intradermal delivery, two different types of gene guns were used, the Acell and the Oxford Bioscience device (PowderJect Pharmaceuticals, Oxford, UK).

ELISPOT assays

CD8+ T cells were quantified in the spleens of immunised mice without *in vitro* restimulation using the peptide epitopes indicated and the ELISPOT assay as described by Miyahara *et al* (1993). Briefly, 96-well nitrocellulose plates (Miliscreen MAHA, Millipore, Bedford UK) were coated with 15 μg/ml of the anti-mouse interferon-γ monoclonal antibody R4 (EACC) in 50 μl of phosphate-buffered saline (PBS). After overnight incubation at 4°C the wells were washed once with PBS and blocked for 1 hour at room temperature with 100 μl RPMI with 10% FCS. Splenocytes from immunised mice were resuspended to 1 x 10^7 cells/ml and placed in duplicate in the antibody coated wells and serially diluted. Peptide was added to each well to a final concentration of 1 μg/ml. Additional wells without peptide were used as a control for peptide-dependence of interferon-γ secretion. After incubation at 37°C in 5%CO_2 for 12-18 hours the plates were washed 6 times with PBS and water. The wells were then incubated for 3 hours at room temperature with a solution of 1 μg/ml of biotinylated anti-mouse interferon-γ monoclonal antibody XMG1.2 (Pharmingen, CA, USA) in PBS. After further washes with PBS, 50 μl of a 1 μg/ml solution of streptavidin-alkaline-phosphatase polymer (Sigma) was added for 2 hours at room temperature. The spots were developed by adding 50 μl of an alkaline phosphatase conjugate substrate solution (Biorad, Hercules, CA, USA). After the appearance of spots the reaction was stopped by washing with water. The number of spots was determined with the aid of a stereomicroscope.

ELISPOT assays on the chimpanzee peripheral blood lymphocytes were performed using a very similar method employing the assay and reagents developed to detect human CD8 T cells (Mabtech, Stockholm).

CTL assays

CTL assays were performed using chromium labelled target cells as indicated and cultured mouse spleen cells as effector cells as described by Allsopp *et al.* (1996). CTL assays using chimpanzee or macaque cells were performed as described for the detection of human CTL by Hill *et al.* (1992) using EBV-transformed autologous chimpanzee chimpanzee or macaque B cell lines as target cells.

P. berghei challenge

Mice were challenged with 2000 (BALB/c) or 200 (C57BL/6) sporozoites of the *P. berghei* ANKA strain in 200 µl RPMI by intravenous inoculation as described (Lanar *et al.* 1996). These sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes maintained at 18°C for 20-25 days after feeding on infected mice. Blood-stage malaria infection, indicating a failure of the immunisation, was detected by observing the appearance of ring forms of *P. berghei* in Giemsa-stained blood smears taken at 5-12 days post-challenge.

P. falciparum challenge

The chimpanzees were challenged with 20,000 *P. falciparum* sporozoites of the NF54 strain dissected from the salivary glands of *Anopheles gambiae* mosquitoes, by intravenous inoculation under anaesthesia. Blood samples from these chimpanzees were examined daily from day 5 after challenge by microscopy and parasite culture, in order to detect the appearance of low levels of *P. falciparum* parasites in the peripheral blood.

P815 tumour challenges

Mice were challenged with 1 x 10^5 P815 cells in 200 µl of PBS by intravenous inoculation. Animals were monitored for survival.

Influenza virus challenges

Mice were challenged with 100 haemagglutinating units (HA) of influenza virus A/PR/8/34 by intranasal inoculation. Following challenge the animals were weighed daily and monitored for survival.

Determining peptide specific CTL using tetramers

Tetrameric complexes consisting of Mamu-A*01-heavy chain and β_2-microglobulin were made as described by Ogg *et al* (1998). DNA coding for the leaderless extracellular portion of the Mamu-A*01 MHC class I heavy chain was PCR-amplified from cDNA using 5'primer MamuNdeI: 5'-CCT GAC TCA GAC CAT ATG GGC TCT CAC TCC ATG [SEQ ID NO: 74] and 3' primer: 5'-GTG ATA AGC TTA ACG ATG ATT CCA CAC CAT TTT CTG TGC ATC CAG AAT ATG ATG CAG GGA TCC CTC CCA TCT CAG GGT GAG GGG C [SEQ ID NO: 75]. The former primer contained a Nde*I* restriction site, the latter included a Hind*III* site and encoded for the bioinylation enzyme BirA substrate peptide. PCR products were digested with Nde*I* and Hind*III* and ligated into the same sites of the polylinker of bacterial expression vector pGMT7. The rhesus monkey gene encoding a leaderless β_2-microglobulin was PCR amplifed from a cDNA clone using primers B2MBACK: 5'-TCA GAC CAT ATG TCT CGC TCC GTG GCC [SEQ ID NO: 76] and B2MFOR: 5'-TCA GAC AAG CTT TTA CAT GTC TCG ATC CCA C [SEQ ID NO: 77] and likewise cloned into the Nde*I* and Hind*III* sites of pGMT7. Both chains were expressed in *E. coli* strain BL-21, purified from inclusion bodies, refolded in the presence of peptide CTPYDINQM [SEQ ID NO: 54], biotinylated using the BirA enzyme (Avidity) and purified with FPLC and monoQ ion exchange columns. The amount of biotinylated refolded MHC-peptide complexes was estimated in an ELISA assay, whereby monomeric complexes were first captured by conformation sensitive monoclonal

antibody W6/32 and detected by alkaline phosphatase (AP) –conjugated streptavidin (Sigma) followed by colorimetric substrate for AP. The formation of tetrameric complexes was induced by addition of phycoerythrin (PE)-conjugated streptavidin (ExtrAvidin; Sigma) to the refolded biotinylated monomers at a molar ratio of MHC-peptide : PE-streptavidin of 4 : 1. The complexes were stored in the dark at 4°C. These tetramers were used to analyse the frequency of Mamu-A*01/gag-specific CD8+ T cells in peripherial blood lymphocytes (PBL) of immunised macaques.

EXAMPLE 2

Immunogenicity Studies in Mice

Previous studies of the induction of CTL against epitopes in the circumsporozoite (CS) protein of *Plasmodium berghei* and *Plasmodium yoelii* have shown variable levels of CTL induction with different delivery systems. Partial protection has been reported with plasmid DNA (Sedegah *et al.* 1994), influenza virus boosted by replicating vaccinia virus (Li *et al.* 1991), adenovirus (Rodrigues et al 1997) and particle delivery systems (Schodel *et al.* 1994). Immunisation of mice intramuscularly with 50 micrograms of a plasmid encoding the CS protein produced moderate levels of CD8+ cells and CTL activity in the spleens of these mice after a single injection (Figures 3, 4).

For comparison groups of BALB/c mice (n = 5) were injected intravenously with 10^6 ffu/pfu of recombinant vaccinia viruses of different strains (WR, NYVAC and MVA) all expressing *P. berghei* CSP. The frequencies of peptide-specific CD8+ T cells were measured 10 days later in an ELISPOT assay. MVA.PbCSP induced 181 +/- 48, NYVAC 221+/- 27 and WR 94+/- 19 (mean +/- standard deviation) peptide-specific CD8+ T cells per million splenocytes. These results show that surprisingly replication-impaired vaccinia viruses are superior to replicating strains in

priming a CD8+ T cell response. We then attempted to boost these moderate CD8+ T cell responses induced by priming with either plasmid DNA or MVA using homologous or heterologous vectors. A low level of CD8+ T cells was observed after two immunisations with CS recombinant DNA vaccine alone, the recombinant MVA vaccine alone or the recombinant MVA followed by recombinant DNA (Figure 3). A very much higher level of CD8+ T cells was observed by boosting the DNA-primed immune response with recombinant MVA. In a second experiment using ten mice per group the enhanced immunogenicity of the DNA/MVA sequence was confirmed: DNA/MVA 856 +/- 201; MVA/DNA 168 +/- 72; MVA/MVA 345+/- 90; DNA/DNA 92 +/- 46. Therefore the sequence of a first immunisation with a recombinant plasmid encoding the CS protein followed by a second immunisation with the recombinant MVA virus yielded the highest levels of CD8+ T lymphocyte response after immunisation.

Figure 3 shows malaria CD8 T cell ELISPOT data following different immunisation regimes. Results are shown as the number of peptide-specific T cells per million splenocytes. Mice were immunised either with the PbCSP-plasmid DNA or the PbCSP-MVA virus or combinations of the two as shown on the X axis, at two week intervals and the number of splenocytes specific for the pb9 malaria epitope assayed two weeks after the last immunisation. Each point represents the number of spot-forming cells (SFCs) measured in an individual mouse. The highest level of CD8+ T cells was induced by priming with the plasmid DNA and boosting with the recombinant MVA virus. This was more immunogenic than the reverse order of immunisation (MVA/DNA), two DNA immunisations (DNA/DNA) or two MVA immunisations (MVA/MVA). It was also more immunogenic than the DNA and MVA immunisations given simultaneously (DNA + MVA 2w), than one DNA immunisation (DNA 4w) or one MVA immunisation given at the earlier or later time point (MVA 2w and MVA 4w).

Figure 4 shows that malaria CD8 T cell ELISPOT and CTL levels are substantially boosted by a recombinant MVA immunisation following priming with a plasmid DNA encoding the same antigen. **A and C.** CD8+ T cell responses were measured in BALB/c mice using the γ-interferon ELISPOT assay on fresh splenocytes incubated for 18 h with the K^d restricted peptide SYIPSAEKI [SEQ ID NO: 67] from *P. berghei* CSP and the L^d restricted peptide TPHPARIGL [SEQ ID NO: 69] from *E. coli* β-galactosidase. Note that the ELISPOT counts are presented on a logarithmic scale. **B and D.** Splenocytes from the same mice were also assayed in conventional ^{51}Cr-release assays at an effector: target ration of 100:1 after 6 days of *in vitro* restimulation with the same peptides (1 μg/ml).

The mice were immunised with plasmid DNA expressing either *P. berghei* CSP and TRAP, *Pb*CSP alone, the malaria epitope cassette including the *P. berghei* CTL epitope (labelled pTH.M), or β-galactosidase. ELISPOT and CTL levels measured in mice 23 days after one DNA immunisation are shown in A and B respectively. The same assays were performed with animals that received additionally $1x\ 10^7$ ffu of recombinant MVA expressing the same antigen(s) two weeks after the primary immunisation. The ELISPOT and CTL levels in these animals are shown in C and D respectively. Each bar represents data from an individual animal.

Studies were also undertaken of the immunogenicity of the epitope string HM comprising both HIV and malaria epitopes in tandem. Using this epitope string again the highest levels of CD8+ T cells and CTL were generated in the spleen when using an immunisation with DNA vaccine followed by an immunisation with a recombinant MVA vaccine (Table 4, Figure 5).

Table 4 Immunogenicity of various DNA/MVA combinations as determined by ELISPOT assays

Immunisation 1	Immunisation 2	HIV epitope	Malaria epitope
DNA-HM	DNA-HM	56 ± 26	4 ± 4
MVA-HM	MVA-HM	786 ± 334	238 ±106
MVA-HM	DNA-HM	306 ± 78	58 ± 18
DNA-HM	MVA-HM	1000 ± 487	748 ± 446
None	DNA-HM	70 ± 60	100 ± 10
None	MVA-HM	422 ± 128	212 ± 94

Table 4 shows the results of ELISPOT assays performed to measure the levels of specific CD8+ T cells to HIV and malaria epitopes following different immunisation regimes of plasmid DNA and MVA as indicated. The numbers are spot-forming cells per million splenocytes. The HM epitope string is illustrated in figure 2. BALB/c mice were used in all cases. The malaria epitope was pb9 as in figures 2 and 3. The HIV epitope was RGPGRAFVTI [SEQ ID NO: 51]. The immunisation doses were 50 μg of plasmid DNA or 10^7 focus-forming units (ffu) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days in all cases.

Figure 5 shows the CTL responses induced in BALB/c mice to malaria and HIV epitopes by various immunisation regimes employing plasmid DNA and recombinant MVA. Mice were immunised intramuscularly as described in the legend to table 3 and in methods. High levels of CTL (>30% specific lysis at effector/target ration of 25:1) were observed to both the malaria and HIV epitopes only after priming with

plasmid DNA and boosting with the recombinant MVA. The antigen used in this experiment is the HIV-malaria epitope string. The recombinant MVA is denoted MVA.HM and the plasmid DNA expressing this epitope string is denoted pTH.HM. Levels of specific lysis at various effector to target ratios are shown. These were determined after 5 days *in vitro* restimulation of splenocytes with the two peptides pb9 and RGPGRAFVTI [SEQ ID NO: 51].

Comparison of numerous delivery systems for the induction of CTL was reported by Allsopp *et al.* (1996). Recombinant Ty-virus like particles (Ty-VLPs) and lipid-tailed malaria peptides both gave good CTL induction but Ty-VLPs were better in that they required only a single immunising dose for good CTL induction. However, as shown here even two doses of Ty particles fail to induce significant protection against sporozoite challenge (Table 7, line 1). Immunisation with a recombinant modified vaccinia Ankara virus encoding the circumsporozoite protein of *P. berghei* also generates good levels of CTL. However, a much higher level of CD8+ T cell response is achieved by a first immunisation with the Ty-VLP followed by a second immunisation with the MVA CS vaccine (Table 5).

Table 5 Immunogenicity of various Ty-VLP/MVA combinations as determined by ELISPOT and CTL assays

Immunisation 1	**Immunisation 2**	**ELISPOT No**	**%Specific Lysis**
Ty-CABDH	Ty- CABDH	75	15
MVA.PbCSP	MVA.PbCSP	38	35
Ty-CABDH	MVA.PbCSP	225	42
Ty- CABDH	MVA.HM	1930	nd

Table 5 Results of ELISPOT and CTL assays performed to measure the levels of specific CD8+ T cells to the malaria epitope pb9 following different immunisation regimes of Ty-VLPs and recombinant MVA virus as indicated. The CTL and ELISPOT data are from different experiments. The ELISPOT levels (spots per million splenocytes) are measured on un-restimulated cells and the CTL activity, indicated as specific lysis at an effector to target ratio of 40:1, on cells restimulated with pb9 peptide *in vitro* for 5-7 days. Both represent mean levels of three mice. BALB/c mice were used in all cases. The immunisation doses were 50 μg of Ty-VLP or 10^7 ffu (foci forming units) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days. MVA.HM includes cassettes CAB.

Priming of an immune response with DNA delivered by a gene gun and boosting with recombinant MVA

Immunogenicity and challenge.

The use of a gene gun to deliver plasmid DNA intradermally and thereby prime an immune response that could be boosted with recombinant MVA was investigated. Groups of BALB/c mice were immunised with the following regimen:

I) Three gene gun immunisations with pTH.PbCSP (4 mg per immunisation) at two week intervals

II) Two gene gun immunisations followed by MVA i.v. two weeks later

III) One intramuscular DNA immunisation followed by MVA i.v. two weeks later.

The immunogenicity of the three immunisation regimens was analysed using ELISPOT assays. The highest frequency of specific T cells was observed with two gene gun immunisations followed by an MVA i.v.

boost and the intramuscular DNA injection followed an MVA i.v. boost (Figure 6).

Figure 6 shows the results of ELISPOT assays performed to measure the levels of specific CD8+ T cells to the malaria epitope pb9 following different immunisation regimes. Groups of BALB/c mice (n= 3) were immunised as indicated (g.g. = gene gun). The time between all immunisations was 14 days. ELISPOT assays were done two weeks after the last immunisation.

CTL induction to the same antigen in different mouse strains

To address the question whether the boosting effect described above in BALB/c mice with two CTL epitopes SYIPSAEKI [SEQ ID NO: 67] derived from *P. berghei* CSP and RGPGRAFVTI [SEQ ID NO: 68] derived from HIV) is a universal phenomenon, two sets of experiments were carried out. CTL responses to the influenza nucleoprotein were studied in five inbred mouse strains. In a first experiment three published murine CTL epitopes derived from the influenza nucleoprotein were studied (see Table 3). Mice of three different H-2 haplotypes, BALB/c and DBA/2 ($H\text{-}2^d$), C57BL/6 and 129 ($H\text{-}2^b$); CBA/J ($H\text{-}2^k$), were used. One set of animals was immunised twice at two week intervals with the plasmid V1J-NP encoding the influenza nucleoprotein. Another set of identical animals was primed with V1J-NP and two weeks later boosted intravenously with 10^6 ffu of MVA.NP, expressing influenza virus NP. The levels of CTL in individual mice were determined in a ^{51}Cr-release assay with peptide re-stimulated splenocytes. As shown in Figure 7, the DNA priming/MVA boosting immunisation regimen induced higher levels of lysis in all the mouse strains analysed and is superior to two DNA injections.

Figure 7 shows the CTL responses against influenza NP in different mouse strains. Mice of different strains were immunised twice two weeks apart with a DNA vaccine V1J-NP encoding for the influenza

nucleoprotein (open circles) or primed with the same DNA vaccine and two weeks later boosted with recombinant MVA expressing influenza virus nucleoprotein (closed circles). Two weeks after the last immunisation splenocytes were restimulated in vitro with the respective peptides (Table 3). The CTL activity was determined in a standard ^{51}Cr-release assay with MHC class I-matched target cells.

CTL induction to different antigens in different mouse strains

The effect of MVA boosting on plasmid DNA-primed immune responses was further investigated using different antigens and different inbred mouse strains. Mice of different strains were immunised with different antigens using two DNA immunisations and compared with DNA/MVA immunisations. The antigens used were *E. coli* -galactosidase, the malaria/HIV epitope string, a murine tumour epitope string and *P. falciparum* TRAP. Compared with two DNA immunisations the DNA-priming/MVA-boosting regimen induced higher levels of CTL in all the different mouse strains and antigen combinations tested (Figure 8).

Figure 8 shows CTL responses against different antigens induced in different inbred mouse strains. Mice were immunised with two DNA vaccine immunisations two weeks apart (open circles) or primed with a DNA vaccine and two weeks later boosted with a recombinant MVA expressing the same antigen (closed circles). The strains and antigens were: C57BL/6; *P. falciparum* TRAP in **A**. DBA/2; *E .coli* β-galactosidase in **B**. BALB/c; HM epitope string CTL activity against malaria peptide (pb9) in **C**. DBA/2; HM epitope string CTL activity against pb9 in **D**. BALB/c; HM epitope string CTL activity against HIV peptide in **E**. DBA/2; HM epitope string CTL activity against HIV peptide in **F**. BALB/c; tumour epitope string CTL activity against P1A-derived peptide in **G**. DBA/2; tumour epitope string CTL activity against P1A-derived peptide in **H**. Sequences of peptide epitopes are shown in table 3. Each curve shows the data for an individual mouse.

Sporozoites can efficiently prime an immune response that is boostable by MVA

Humans living in malaria endemic areas are continuously exposed to sporozoite inoculations. Malaria-specific CTL are found in these naturally exposed individuals at low levels. To address the question whether low levels of sporozoite induced CTL responses can be boosted by MVA, BALB/c mice were immunised with irradiated (to prevent malaria infection) *P. berghei* sporozoites and boosted with MVA. Two weeks after the last immunisation splenocytes were re-stimulated and tested for lytic activity. Two injections with 50 or 300 + 500 sporozoites induced very low or undetectable levels of lysis. Boosting with MVA induced high levels of peptide specific CTL. MVA alone induced only moderate levels of lysis (Figure 9).

Figure 9 shows sporozoite-primed CTL responses are substantially boosted by MVA. Mice were immunised with two low doses (50 + 50) of irradiated sporozoites in **A**. two high doses (300 +500) of sporozoites in **B**; mice were boosted with MVA.PbCSP following low-dose sporozoite priming in **D**; high dose sporozoite priming in **E**. CTL responses following immunisation with MVA.PbCSP are shown in **C**.

Recombinant adenoviruses as priming agent

The prime-boost immunisation regimen has been exemplified using plasmid DNA and recombinant Ty-VLP as priming agent. Here an example using non-replicating adenoviruses as the priming agent is provided. Replication-deficient recombinant Adenovirus expressing *E. coli* β-galactosidase (Adeno-GAL) was used. Groups of BALB/c mice were immunised with plasmid DNA followed by MVA or with Adenovirus followed by MVA. All antigen delivery systems used encoded *E. coli* β-galactosidase. Priming a CTL response with plasmid DNA or Adenovirus and boosting with MVA induces similar levels of CTL (Figure 10).

Figure 10 shows CTL responses primed by plasmid DNA or recombinant Adenovirus and boosted with MVA . Groups of BALB/c mice (n=3) were primed with plasmid DNA **A** or recombinant Adenovirus expressing β-galactosidase **B**. Plasmid DNA was administered intramuscularly, MVA intravenously and Adenovirus intradermally. Splenocytes were restimulated with peptide TPHPARIGL [SEQ ID NO: 69] two weeks after the last immunisation. CTL activity was tested with peptide-pulsed P815 cells.

Immunogenicity of the DNA prime vaccinia boost regimen depends on the replication competence of the strain of vaccinia virus used

The prime boosting strategy was tested using different strains of recombinant vaccina viruses to determine whether the different strains with strains differing in their replication competence may differ in their ability to boost a DNA-primed CTL response. Boosting with replication-defective recombinant vaccinia viruses such as MVA and NYVAC resulted in the induction of stronger CTL responses compared to CTL responses following boosting with the same dose of replication competent WR vaccinia virus (Figure 11).

Figure 11 shows CTL responses in BALB/c mice primed with plasmid DNA followed by boosting with different recombinant vaccinia viruses. Animals were primed with pTH.PbCSP 50 μg/mouse i.m. and two weeks later boosted with different strains of recombinant vaccina viruses (10^6 pfu per mouse i.v.) expressing PbCSP. The different recombinant vaccinia virus strains were MVA in A; NYVAC in B and WR in C. The superiority of replication-impaired vaccinia strains over replicating strains was found in a further experiment. Groups of BALB/c mice (n = 6) were primed with 50μg/animal of pSG2.PbCSP (i.m.) and 10 days later boosted i.v. with 10^6 ffu/pfu of recombinant MVA, NYVAC and WR expressing PbCSP. The frequencies of peptide-specific CD8+ T cells were determined using the ELISPOT assay. The frequencies were: MVA 1103

+/- 438, NYVAC 826 +/- 249 and WR 468 +/- 135. Thus using both CTL assays and ELISPOT assays as a measure of CD8 T cell immunogenicity a surprising substantially greater immunogenicity of the replication-impaired vaccinia strains was observed compared to the replication competent strain.

The use of recombinant canary or fowl pox viruses for boosting CD8+ T cell responses

Recombinant canary pox virus (rCPV) or fowl pox virus (rFVP) are made using shuttle vectors described previously (Taylor *et al.* Virology 1992, 187: 321-328 and Taylor *et al.* Vaccine 1988, 6: 504-508). The strategy for these shuttle vectors is to insert the gene encoding the protein of interest preceded by a vaccinia-specific promoter between two flanking regions comprised of sequences derived from the CPV or FPV genome. These flanking sequences are chosen to avoid insertion into essential viral genes. Recombinant CPV or FPV are generated by *in vivo* recombination in permissive avian cell lines i.e. primary chicken embryo fibroblasts. Any protein sequence of antigens or epitope strings can be expressed using fowl pox or canary pox virus. Recombinant CPV or FPV is characterised for expression of the protein of interest using antigen-specific antibodies or including an antibody epitope into the recombinant gene. Recombinant viruses are grown on primary CEF. An immune response is primed using plasmid DNA as described in Materials and Methods. This plasmid DNA primed immune response is boosted using 10^7 ffu/pfu of rCPV or rFPV inoculated intravenously, intradermally or intramuscularly. CD8+ T cell responses are monitored and challenges are performed as described herein.

EXAMPLE 3

Malaria Challenge Studies in Mice

To assess the protective efficacy of the induced levels of CD8+ T cell response immunised BALB/c or C57BL/6 mice were challenged by intravenous injection with 2000 or 200 *P. berghei* sporozoites. This leads to infection of liver cells by the sporozoites. However, in the presence of a sufficiently strong T lymphocyte response against the intrahepatic parasite no viable parasite will leave the liver and no blood-stage parasites will be detectable. Blood films from challenged mice were therefore assessed for parasites by microscopy 5-12 days following challenge.

BALB/c mice immunised twice with a mixture of two plasmid DNAs encoding the CS protein and the TRAP antigen, respectively, of *P. berghei* were not protected against sporozoite challenge. Mice immunised twice with a mixture of recombinant MVA viruses encoding the same two antigens were not protected against sporozoite challenge. Mice immunised first with the two recombinant MVAs and secondly with the two recombinant plasmids were also not protected against sporozoite challenge. However, all 15 mice immunised first with the two plasmid DNAs and secondly with the two recombinant MVA viruses were completely resistant to sporozoite challenge (Table 6 A and B).

To assess whether the observed protection was due to an immune response to the CS antigen or to TRAP or to both, groups of mice were then immunised with each antigen separately (Table 6 B). All 10 mice immunised first with the CS plasmid DNA and secondly with the CS MVA virus were completely protected against sporozoite challenge. Fourteen out of 16 mice immunised first with the TRAP plasmid DNA vaccine and secondly with the TRAP MVA virus were protected against sporozoite challenge. Therefore the CS antigen alone is fully protective

when the above immunisation regime is employed and the TRAP antigen is substantially protective with the same regime.

The good correlation between the induced level of CD8+ T lymphocyte response and the degree of protection observed strongly suggests that the CD8+ response is responsible for the observed protection. In previous adoptive transfer experiments it has been demonstrated that CD8+ T lymphocyte clones against the major CD8+ T cell epitope in the *P. berghei* CS protein can protect against sporozoite challenge. To determine whether the induced protection was indeed mediated by CD8+ T cells to this epitope we then employed a plasmid DNA and a recombinant MVA encoding only this nine amino acid sequence from *P. berghei* as a part of a string of epitopes (Table 6 B). (All the other epitopes were from micro-organisms other than *P. berghei).* Immunisation of 10 mice first with a plasmid encoding such an epitope string and secondly with a recombinant MVA also encoding an epitope string with the *P. berghei* CTL epitope led to complete protection from sporozoite challenge (Table 6 B). Hence the induced protective immune response must be the CTL response that targets this nonamer peptide sequence.

Table 6 Results of mouse challenge experiments using different combinations of DNA and MVA vaccine

Immunisation 1	Immunisation 2	No. Infected/ No. challenged	%Protection
A.	Antigens used: PbCSP + PbTRAP		
DNA	DNA	5/5	0%
MVA	MVA	9/10	10%
DNA	MVA	0/5	100%
MVA	DNA	5/5	0%
	Control mice immunised with β-galactosidase		
DNA	DNA	5/5	0%
MVA	MVA	5/5	0%
DNA	MVA	5/5	0%
MVA	DNA	5/5	0%
B.			
DNA (CSP +TRAP)	MVA (CSP +TRAP)	0/10	100%
DNA (CSP)	MVA (CSP)	0/10	100%
DNA (TRAP)	MVA (TRAP)	2/16	88%
DNA (epitope)	MVA (epitope)	0/11	100%
DNA (beta-gal)	MVA (beta-gal)	6/7	14%
none	none	9/10	10%

Table 6 Results of two challenge experiments (A and B) using different immunisation regimes of plasmid DNA and MVA as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 μg of plasmid DNA or 10^6 ffu of recombinant MVA. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP and TRAP indicate the entire *P. berghei* antigen and 'epitope' indicates the cassettes of epitopes shown in table 1 containing

only a single *P. berghei* K^d-restricted nonamer CTL epitope. Note that in experiment B immunisation with the epitope string alone yields 100% protection

Mice immunised twice with recombinant Ty-VLPs encoding pb9 were fully susceptible to infection. Similarly mice immunised twice with the recombinant MVA encoding the full CS protein were fully susceptible to infection. However, the mice immunised once with the Ty-VLP and subsequently once with the recombinant MVA showed an 85% reduction in malaria incidence when boosted with MVA expressing the full length CS protein, and 95% when MVA expressing the HM epitope string which includes pb9 was used to boost (Table 7).

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA

Immunisation 1	Immunisation 2	No. Infected/No.challenged	%Protection
Ty-CABDHFE	Ty- CABDHFE	7/8	13%
Ty-CABDH	MVA.PbCSP	2/13	85%
Ty- CABDHFE	MVA-NP	5/5	0%
MVA.PbCSP	MVA.PbCSP	6/6	0%
MVA.HM	Ty- CABDHFE	14/14	0%
Ty- CABDHFE	MVA.HM	1/21	95%
none	MVA.HM	8/8	0%
none	none	11/12	9%

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA as indicated. BALB/c mice were used in all cases. Immunisations were of 50 μg of Ty-VLP or 10^7 ffu of recombinant MVA administered intravenously. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of

2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP indicates the entire *P. berghei* antigen. Ty-VLPs carried epitope cassettes CABDH or CABDHFE as described in table 1. MVA.HM includes cassettes CAB.

To determine whether the enhanced immunogenicity and protective efficacy observed by boosting with a recombinant MVA is unique to this particular vaccinia virus strain or is shared by other recombinant vaccinias the following experiment was performed. Mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with either (i) recombinant MVA encoding this antigen; (ii) recombinant wild-type vaccinia virus (Western Reserve strain) encoding the same antigen (Satchidanandam *et al.* 1991), or (iii) recombinant NYVAC (COPAK) virus (Lanar *et al.* 1996) encoding the same malaria antigen. The highest degree of protection was observed with boosting by the MVA recombinant, 80% (Table 8). A very low level of protection (10%) was observed by boosting with the wild-type recombinant vaccinia virus and a significant level of protection, 60%, by boosting with the NYVAC recombinant. Hence the prime-boost regime we describe induces protective efficacy with any non-replicating vaccinia virus strain. Both the MVA recombinant and NYVAC were significantly ($P < 0.05$ for each) better than the WR strain recombinant.

Table 8 Challenge data results for DNA boosted with various vaccinia strain recombinants.

Immunisation 1	Immunisation 2	No. Infected/No. challenged	%Protection
DNA-beta gal.	MVA.NP	8/8	0%
DNA-CSP	MVA-CSP	2/10	80%
DNA-CSP	WR-CSP	9/10	10%
DNA-CSP	NYVAC-CSP	4/10	60%

Table 8 Results of a challenge experiment using different immunisation regimes of plasmid DNA and various vaccinia recombinants as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 μg of plasmid DNA or 10^6 ffu/pfu of recombinant MVA or 10^4 ffu/pfu of recombinant wild type (WR) vaccinia or 10^6 ffu/pfu of recombinant NYVAC. Because the WR strain will replicate in the host and the other strains will not, in this experiment a lower dose of WR was used. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. pbCSP indicates the entire *P. berghei* antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The first immunisation of group A mice was with the plasmid DNA vector expressing beta galactosidase but no malaria antigen.

In a further experiment shown in Table 8, mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with either (i) recombinant MVA encoding this antigen; (ii) recombinant WR vaccinia virus encoding the same antigen or (iii) recombinant NYVAC (COPAK) virus encoding the same malaria antigen, all at 10^6 ffu/pfu. A high and statistically significant degree of protection was observed with boosting with recombinant NYVAC (80%) or recombinant MVA (66%). A low and non-significant level of protection (26%) was observed by boosting with the WR recombinant vaccinia virus (Table 9). MVA and NYVAC boosting each gave significantly more protection than WR boosting ($P = 0.03$ and $P = 0.001$ respectively). These data re-emphasise that non-replicating pox virus strains are better boosting agents for inducing high levels of protection.

Table 9 Influence of different recombinant vaccinia strains on protection.

Immunisation 1 DNA	Immunisation 2	No. inf./ No. chall.	% protection
CSP	MVA.PbCSP	5/15	66
CSP	NYVAC.PbCSP	2/15	80
CSP	WR.PbCSP	11/15	26
β- galactosidase	MVA.NP	8/8	0

Table 9 Results of challenge experiments using different immunisation regimes of plasmid DNA and replication incompetent vaccinia recombinants as boosting immunisation. BALB/c mice were used in all cases. The immunisation doses were 50 μg of plasmid DNA or 10^6 ffu/pfu of recombinant MVA or recombinant wild type (WR) vaccinia or recombinant NYVAC. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. PbCSP indicates the entire *P. berghei* antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The control immunisation was with a plasmid DNA vector expressing β-galactosidase followed by MVA.NP.

Alternative routes for boosting immune responses with recombinant MVA

Intravenous injection of recombinant MVA is not a preferred route for immunising humans and not feasible in mass immunisations.

Therefore different routes of MVA boosting were tested for their immunogenicity and protective efficacy.

Mice were primed with plasmid DNA i.m. Two weeks later they were boosted with MVA administered via the following routes: intravenous (i.v.), subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.p.) and intradermal (i.d.). Two weeks after this boost peptide-specific CD8+ T cells were determined in an ELISPOT assay. The most effective route which induced the highest levels were i.v. and i.d inoculation of MVA. The other routes gave moderate to poor responses (Figure 12).

Figure 12 shows frequencies of peptide-specific CD8+ T cells following different routes of MVA boosting. Results are shown as the number of spot-forming cells (SFC) per one million splenocytes. Mice were primed with plasmid DNA and two weeks later boosted with MVA via the indicated routes. The number of splenocytes specific for the SYIPSAEKI [SEQ ID NO: 67] peptide was determined in INF-γ ELISPOT assays two weeks after the last immunisation. Each bar represents the mean number of SFCs from three mice assayed individually.

Boosting via the i.v. route was compared with the i.d. and i.m route in a challenge experiment. The i.d route gave high levels of protection (80% protection). In the group of animals that were boosted via the i.m. route, 50% of the animals were protected. Complete protection was achieved with MVA boost administered i.v. (Table 10)

Table 10 Influence of the route of MVA administration on protective efficacy

Immunisation 1 DNA	Immunisation 2 MVA	No. infected/ No. challenged	% protection
CSP	CSP i.v.	*0/20	100
CSP	CSP i.d	2/10	80
CSP	CSP i.m.	5/10	50

Epitope	epitope i.v.	1/10	90
NP	NP i.v.	10/10	0

* culminative data from two independent experiments

Table 10 Results from challenge experiments using different routes of MVA boosting immunisation. Animals were primed by intramuscular plasmid DNA injection and two weeks later boosted with the indicated recombinant MVA (10^6 ffu/mouse) administered via the routes indicated. The mice were challenged 16 days after the last immunisation with 2000 P. berghei sporozoites and screened for blood stage parasitemia at day 8 and 10 post challenge. Epitope indicates the polypeptide string HM.

Alternative routes of DNA priming: The use of a gene gun to prime peptide specific CD8+ T cells

Gene gun delivery is described in detail in for example in Eisenbraun *et al.* DNA Cell Biol. 1993, 12: 791-797 and Degano *et al.* Vaccine 1998, 16: 394-398.

The mouse malaria challenge experiments described so far using plasmid DNA to prime an immune response used intramuscular injection of plasmid DNA. Intradermal delivery of plasmid DNA using a biolistic device is another route to prime specific CTL responses. Plasmid DNA is coated onto gold particles and delivered intradermally with a gene gun. Groups of mice (n=10) were immunised three times at two weeks intervals with the gene gun alone (4 μg/immunisation), immunised two times with the gene gun followed by an intravenous MVA.PbCSP boost or immunised intramuscularly with 50 μg of pTH.PbCSP and two weeks later boosted with MVA.PbCSP intravenously. Two weeks after the last immunisation the animals were challenged with 2000 sporozoites to assess protective efficacy of each immunisation regimen. In the group that received the intravenous MVA boost following two gene gun immunisations

one out of ten animals developed blood stage parasitemia (90% protection). Complete protection was observed with intramuscular DNA priming followed by MVA i.v boosting. Seven out of 10 animals that were immunised three times with the gene gun were infected. (30% protection) (Table 11).

Immunisation 1 DNA	Immunisation 2	Immunisation 3	No. inf./ No. chall.	% protection
gene gun DNA	gene gun DNA	gene gun DNA	7/10	30
gene gun DNA	gene gun DNA	MVA.PbCSP	1/10	90
-	DNA i.m	MVA.PbCSP	0/10	100
Naïve			10/10	0

Table 11 Results of challenge experiments comparing different routes of DNA priming (intradermally by gene gun versus intramuscular needle injection). Groups of BALB/c mice (n=10) were immunised as indicated. Each gene gun immunisation delivered 4 μg of plasmid DNA intraepidermally. For i.m. immunisations 50 μg of plasmid DNA were injected. Twenty days after the last immunisation mice were challenged as described previously.

Highly susceptible C57BL/6 mice are protected

C57BL/6 mice are very susceptible to *P. berghei* sporozoite challenge. C57BL/6 mice were immunised using the DNA-MVA prime boost regime with both pre-erythrocytic antigens PbCSP and PbTRAP, and challenged with either 200 or 1000 infectious sporozoites per mouse. (Two hundred sporozoites corresponds to more than twice the dose required to induce infection in this strain). All ten mice challenged with 200 sporozoites showed sterile immunity. Even the group challenged with 1000

sporozoites, 60% of the mice were protected (Table 12). All the naïve C57BL/6 mice were infected after challenge.

Table 12 Protection of C57BL/6 mice from sporozoite challenge

	No. animals inf./ No. challenged	% protection
1000 sporozoites		
DNA followed by MVA	4/10	60
Naïve	5/5	0
200 sporozoites		
DNA followed by MVA	0/10	100
Naïve	5/5	0

Table 12 Results of a challenge experiment using C57BL/6 mice. Animals were immunised with PbCSP and PbTRAP using the DNA followed by MVA prime boost regime. Fourteen days later the mice were challenged with *P. berghei* sporozoites as indicated.

EXAMPLE 4

Protective efficacy of the DNA-priming/MVA-boosting regimen in two further disease models in mice

Following immunogenicity studies, the protective efficacy of the DNA-priming MVA-boosting regimen was tested in two additional murine challenge models. The two challenge models were the P815 tumour model and the influenza A virus challenge model. In both model systems CTL have been shown to mediate protection.

P815 tumour challenges:

Groups (n = 10) of DBA/2 mice were immunised with a combination of DNA followed by MVA expressing a tumour epitope string or the HM epitope

string. Two weeks after the last immunisation the mice were challenged intravenously with 10^5 P815 cells. Following this challenge the mice were monitored regularly for the development of tumour-related signs and survival.

Figure 13 shows the survival rate of the two groups of mice. Sixty days after challenge eight out of ten mice were alive in the group immunised with the tumour epitopes string. In the group immunised with the HM epitope string only 2 animals survived. This result is statistically significant: 2/10 vs 8/10 chi-squared = 7.2. P = 0.007. The onset of death in the groups of animals immunised with the tumour epitope string is delayed compared to the groups immunised with the HM epitope string.

Influenza virus challenges:

Groups of BALB/c mice were immunised with three gene gun immunisations with plasmid DNA, two intramuscular plasmid DNA injections, one i.m. DNA injection followed by one MVA.NP boost i.v. or two gene gun immunisations followed by one MVA.NP boost i.v. Plasmid DNA and recombinant MVA expressed the influenza virus nucleoprotein. Two weeks after the last immunisation the mice were challenged intranasally with 100 HA of influenza A/PR/8/34 virus. The animals were monitored for survival daily after challenge.

Complete protection was observed in the following groups of animals

- two DNA gene gun immunisations followed by one MVA.NP boost i.v.,
- one i.m. DNA injection followed by one MVA.NP boost i.v.
- two i.m. DNA injections.

In the group of animals immunised three times with the gene gun 71% of the animals survived (5/7) and this difference from the control group was not significant statistically ($P > 0.05$). In the naive group 25% of the animals survived (Figure 14) and this group differed significantly ($P < 0.05$) for the two completely protected groups.

Figure 14 shows results of an influenza virus challenge experiment. BALB/c mice were immunised as indicated. GG = gene gun immunisations, im = intramuscular injection, iv = intravenous injection. Survival of the animals was monitored daily after challenge.

In a second experiment groups of 10 BALB/c mice were immunised with MVA.NP i.v. alone, three times with the gene gun, two times with the gene gun followed by one MVA.NP boost i.v. and two i.m injections of V1J-NP followed by one MVA.NP boost. Two weeks after the last immunisation the mice were challenged with 100 HA units of influenza A/PR/8/34 virus.

Complete and statistically significant protection was observed in the following groups of animals:

- two gene gun immunisations followed by one MVA.NP boost,
- two i.m injections of V1J-NP followed by one MVA.NP boost.

In the group receiving one MVA.NP i.v., 30% (3 out of 10) of animals survived. In the group immunised with a DNA vaccine delivered by the gene gun three times, 70% of the animals were protected but this protection was not significantly different from the naïve controls. In this challenge experiment 40% (4 out of 10) of the naive animals survived the challenge.

EXAMPLE 5

Immunogenicity studies in non-human primates

Immunogenicity and protective efficacy of the prime boost regimen in non-human primates.

In order to show that the strong immunogenicity of the DNA priming/MVA boosting regime observed in mice translates into strong immunogenicity in primates, the regimen was tested in macaques. The vaccine consisted of a string of CTL epitopes derived from HIV and SIV sequences (Figure 2), in plasmid DNA or MVA, denoted DNA.H and

MVA.H respectively. The use of defined CTL epitopes in a polyepitope string allows testing for SIV specific CTL in macaques. Due to the MHC class I restriction of the antigenic peptides, macaques were screened for their MHC class I haplotype and Mamu-A*01-positive animals were selected for the experiments described.

Three animals (CYD, DI and DORIS) were immunised following this immunisation regimen:

week 0	DNA (8μg, i.d., gene gun)
week 8	DNA (8μg, i.d., gene gun)
week 17	MVA (5×10^8 pfu, i.d.)
week 22	MVA (5×10^8 pfu, i.d.)

Blood from each animal was drawn at weeks 0, 2, 5, 8, 10, 11, 17, 18, 19, 21, 22, 23, 24 and 25 of the experiment. The animals were monitored for induction of CTL using two different methods. PBMC isolated from each bleed were re-stimulated *in vitro* with a peptide encoded in the epitope string and tested for their ability to recognise autologous peptide-loaded target cells in a chromium release cytotoxicity assay. Additionally, freshly isolated PBMC were stained for antigen specific CD8+ T cells using tetramers.

Following two gene gun immunisations very low levels of CTL were detected using tetramer staining (Figure 15). Two weeks after the first MVA boosting, all three animals developed peptide specific CTL as detected by tetramer staining (Figure 15). This was reflected by the detection of moderate CTL responses following *in vitro* restimulation (Figure 16, week 19). The second boost with MVA.H induced very high levels of CD8+, antigen specific T cells (Figure 15) and also very high levels of peptide specific cytotoxic T cells (Figure 16, week 23).

Figure 15 shows detection of SIV-specific MHC class I-restricted CD8+ T cells using tetramers. Three Mamu-A*A01-positive

macaques were immunised with plasmid DNA (gene gun) followed by MVA boosting as indicated. Frequencies of Mamu-A*A01/CD8 double-positive T cells were identified following FACS analysis. Each bar represents the percentage of CD8+ T cells specific for the Mamu-A*01/gag epitope at the indicated time point. One percent of CD8 T cells corresponds to about $5000/10^6$ peripheral blood lymphocytes. Thus the levels of epitope-specific CD8 T cells in the peripheral blood of these macaques are at least as high as the levels obvserved in the spleens of immunised and protected mice in the malaria studies.

Figure 16 shows CTL induction in macaques following DNA/MVA immunisation. PBMC from three different macaques (CYD, DI and DORIS) were isolated at week 18, 19 and 23 and were restimulated with peptide CTPYDINQM [SEQ ID NO: 54] *in vitro*. After two restimulations with peptide CTPYDINQM [SEQ ID NO: 54] the cultures were tested for their lytic activity on peptide-pulsed autologous target cells. Strong CTL activity was observed.

EXAMPLE 6

Immunogenicity and Challenge Studies in Chimpanzees

To show that a similar regime of initial immunisation with plasmid DNA and subsequent immunisation with recombinant MVA can be effective against *Plasmodium falciparum* malaria in higher primates an immunisation and challenge study was performed with two chimpanzees. Chimp H1 received an initial immunisation with 500 μg of a plasmid expressing Plasmodium falciparum TRAP from the CMV promoter without intron A, CMV-TRAP. Chimp H2 received the same dose of CMV-LSA-1, which expresses the C-terminal portion of the LSA-1 gene of *P. falciparum*. Both chimps received three more immunisations over the next 2 months, but with three plasmids at each immunisation. H1 received CMV-TRAP as before, plus pTH-TRAP, which expresses TRAP using the CMV promoter

with intron A, leading to a higher expression level. H1 also received RSV-LSA-1, which expresses the C-terminal portion of LSA-1 from the RSV promoter. H2 received CMV-LSA-1, pTH-LSA-1 and RSV-TRAP at the second, third and fourth immunisations. The dose was always 500 μg of each plasmid.

It was subsequently discovered that the RSV plasmids did not express the antigens contained within them, so H1 was only immunised with plasmids expressing TRAP, and H2 with plasmids expressing LSA-1.

Between and following these DNA immunisations assays of cellular immune responses were performed at several time points, the last assay being performed at three months following the fourth DNA immunisation, but no malaria-specific T cells were detectable in either ELISPOT assays or CTL assays for CD8+ T cells.

Both animals were subsequently immunised with three doses of 10^8 ffu of a recombinant MVA virus encoding the *P. falciparum* TRAP antigen over a 6 week period. Just before and also following the third recombinant MVA immunisation T cell responses to the TRAP antigen were detectable in both chimpanzees using an ELISPOT assay to whole TRAP protein bound to latex beads. This assay detects both CD4+ and CD8+ T cell responses. Specific CD8+ T responses were searched for with a series of short 8-11 amino acid peptides in both immunised chimpanzees. Such analysis for CD8+ T cell responses indicated that CD8+ T cells were detectable only in the chimpanzee H1. The target epitope of these CD8+ T lymphocytes was an 11 amino acid peptide from TRAP, tr57, of sequence KTASCGVWDEW [SEQ ID NO: 78]. These CD8+ T cells from H1 had lytic activity against autologous target cells pulsed with the tr57 peptide and against autologous target cells infected with the recombinant PfTRAP-MVA virus. A high precursor frequency of these specific CD8+ T cells of about 1 per 500 lymphocytes was detected

in the peripheral blood of this chimpanzee H1 using an ELISPOT assay two months following the final MVA immunisation. No specific CD8+ T cell response was clearly detected in the chimpanzee H2, which was not primed with a plasmid DNA expressing TRAP.

Two months after the third PfTRAP-MVA immunisation challenge of H1 and H2 was performed with 20,000 sporozoites, a number that has previously been found to yield reliably detectable blood stage infection in chimpanzees 7 days after challenge (Thomas *et al.* 1994 and unpublished data). The challenge was performed with the NF54 strain of *Plasmodium falciparum.* This is of importance because the TRAP sequence in the plasmid DNA and in the recombinant MVA is from the T9/96 strain of *P. falciparum* which has numerous amino acid differences to the NF54 TRAP allele (Robson *et al.* 1990). Thus, this sporozoite challenge was performed with a heterologous rather than homologous strain of parasite. In the chimpanzee H2 parasites were detectable in peripheral blood as expected 7 days after sporozoite challenge using *in vitro* parasite culture detection. However, in H1 the appearance of blood stage parasites in culture from the day 7 blood samples was delayed by three days consistent with some immune protective effect against the liver-stage infection. In studies of previous candidate malaria vaccines in humans a delay in the appearance of parasites in the peripheral blood has been estimated to correspond to a substantial reduction in parasite density in the liver (Davis *et al.* 1989). Thus the chimpanzee HI, immunised first with *P. falciparum* TRAP plasmid DNA and subsequently with the same antigen expressed by a recombinant MVA virus showed a strong CD8+ T lymphocyte response and evidence of some protection from heterologous sporozoite challenge.

DISCUSSION

These examples demonstrate a novel regime for immunisation against malaria which induces high levels of protective CD8+ T cells in rodent models of human malaria infection. Also demonstrated is an unprecedented complete protection against sporozoite challenge using subunit vaccines (36 out of 36 mice protected in Table 6 using DNA priming and MVA boosting with the CS epitope containing vaccines). Induction of protective immune responses using the DNA priming/MVA boosting regimen was demonstrated in two additional mouse models of viral infection influenza A model and cancer (P815 tumour model). More importantly for vaccines for use in humans this immunisation regimen is also highly immunogenic for CD8+ T cells in primates. Strong SIV-gag-specific CTL were induced in 3 out of 3 macaques with plasmid DNA and MVA expressing epitope strings. The levels induced are comparable to those found in SIV-infected animals. The data from the chimpanzee studies indicate that the same immunisation regime can induce a strong CD8+ T lymphocyte response against *P. falciparum* in higher primates with some evidence of protection against *P. falciparum* sporozoite challenge.

Ty-VLPs have previously been reported to induce good levels of CD8+ T cell responses against the *P. berghei* rodent malaria (Allsopp *et al.* 1995) but alone this construct is not protective. It has now been found that subsequent immunisation with recombinant MVA boosts the CD8+ T cell response very substantially and generates a high level of protection (Table 7).

Recombinant MVA viruses have not been assessed for efficacy as malaria vaccines previously. Recombinant MVA alone was not significantly protective, nor was priming with recombinant MVA followed by a second immunisation with recombinant plasmid DNA. However, a second immunisation with the recombinant MVA following an initial immunisation with either Ty-VLPs or plasmid DNA yielded impressive

levels of protection. Non-recombinant MVA virus has been safely used to vaccinate thousands of human against smallpox and appears to have an excellent safety profile. The molecular basis of the increased safety and immunogenicity of this strain of vaccinia virus is being elucidated by detailed molecular studies (Meyer *et al.* 1991; Sutter *at al.* 1994).

Plasmid DNA has previously been tested as a malaria vaccine for the *P. yoelii* rodent malaria. High levels of, but not complete, protection is seen in some strains but in other strains of mice little or no protection was observed even after multiple immunisations (Doolan *et al.* 1996). Although plasmid DNA has been proposed as a method of immunisation against *P. falciparum,* success has not previously been achieved. The evidence provided here is the first evidence to show that plasmid DNA may be used in an immunisation regime to induce protective immunity against the human malaria parasite *P. falciparum.*

A similar regime of immunisation to the regime demonstrated herein can be expected to induce useful protective immunity against *P. falciparum* in humans. It should be noted that five of the vaccine constructs employed in these studies to induce protective immunity in rodents or chimpanzees contain *P. falciparum* sequences and could therefore be used for human immunisation against *P. falciparum.* These are: 1. The *P. falciparum* TRAP plasmid DNA vaccine. 2. The *P. falciparum* TRAP recombinant MVA virus. 3. The Ty-VLP encoding an epitope string of numerous *P falciparum* epitopes, as well as the single *P. berghei* CTL epitope. 4. The plasmid DNA encoding the same epitope string as 3. 5. The recombinant MVA encoding the longer HM epitope string including many of the malaria epitopes in 3 and 4. Similarly the plasmid DNAs and MVA encoding HIV epitopes for human class I molecules could be used in either prophylactic or therapeutic immunisation against HIV infection.

These studies have provided clear evidence that a novel sequential immunisation regime employing a non-replicating or replication-impaired pox virus as a boost is capable of inducing a strong protective CD8+ T cell response against the malaria parasite. The examples demonstrate clearly a surprising and substantial enhancement of CD8+ T cell responses and protection compared to replicating strains of pox viruses. Because there is no reason to believe that the immunogenicity of CD8+ T cell epitopes from the malaria parasite should differ substantially from CD8+ T cell epitopes in other antigens it is expected that the immunisation regime described herein will prove effective at generating CD8+ T cell responses of value against other diseases. The critical step in this immunisation regimen is the use of non-replicating or replication-impaired recombinant poxviruses to boost a pre-existing CTL response. We have shown that CTL responses can be primed using different antigen delivery systems such as a DNA vaccine i.d. and i.m, a recombinant Ty-VLP, a recombinant adenovirus and irradiated sporozoites. This is supported by the data presented on the generation of a CD8+ T cell response against HIV, influenza virus and tumours. Amongst several known examples of other diseases against which a CD8+ T cell immune response is important are the following: infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and *Trypanosoma*. Induction of protective CTL responses against influenza A virus has been demonstrated in Figure 14. Furthermore, the immunisation regime described herein is expected to be of value in immunising against forms of cancer where CD8+ T cell responses plays a protective role. The induction of protective CTL responses using the DNA prime MVA boost regime against tumours is

shown in Figure 13. Specific examples in humans include melanoma, cancer of the breast and cancer of the colon.

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5

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12, line 10 - 13.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

European Collection of Animal Cell Cultures (CAMR)

Address of depositary institution *(including postal code and country)*

Salisbury
Wiltshire
SP4 0JG
United Kingdom

Date of deposit	Accession Number
5 June 1997	V97060511

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☒

In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), UK Patent Rules 1995, Schedule 2, Paragraph 3, Australian Regulation 3.25(3), Danish

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☒ This sheet was received by the International Bureau on: 07 SEPTEMBER 1998
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

C. ADDITIONAL INDICATIONS (continued)

Patents Act Sections 22 and 33(3), Icelandic Patents Act Sections 22 and 33(3), Norwegian Patents Act Sections 22 and 33(3) and generally similar provisions *mutatis mutandis* for any other designated State.

CLAIMS

1. A kit for generating a protective CD8+ T cell immune response against at least one target antigen, which kit comprises:

(i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and

(ii) a boosting composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;

with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.

2. The kit according to claim 1, wherein the source of CD8+ T cell epitopes in (i) is a non-viral vector or a non-replicating or replication-impaired viral vector.

3. The kit according to claim 1 or claim 2, wherein the source of CD8+ T cell epitopes in (i) is not a poxvirus vector.

4. The kit according to claim 2 or claim 3, wherein the source of CD8+ T cell epitopes in (i) is DNA or RNA.

5. The kit according to claim 4, wherein the source of epitopes in (i) is a recombinant DNA plasmid.

6. The kit according to claim 4 or claim 5, further comprising GM-CSF as an adjuvant for (i).

7. The kit according to any one of claims 1 to 6, wherein the source of CD8+ T cell epitopes in (i) encodes or comprises the target antigen.

8. The kit according to any one of claims 4 to 6, wherein the source of epitopes in (i) encodes a single CD8+ T cell epitope or a recombinant string of two or more CD8+ T cell epitopes.

9. The kit according to any one of claims 1 to 3, wherein the source of epitopes in (i) is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen.

10. The kit according to claim 9, wherein the source of CD8+ T cell epitopes in (i) is a recombinant protein particle such as a Ty virus-like particle (VLP).

11. The kit according to any one of claims 1 to 3, wherein the source of epitopes in (i) is a recombinant adenovirus vector.

12. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant vaccinia virus vector.

13. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the vaccinia virus strain Modified Virus Ankara (MVA), or a strain derived therefrom.

14. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the strain NYVAC or a strain derived therefrom.

15. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant avipox vector such as canary pox or fowl pox or strains derived therefrom such as ALVAC.

16. The kit according to any one of claims 1 to 15, for generating a protective immune response against a pathogen or tumour comprising the target antigen.

17. The kit according to claim 16, for generating a protective immune response against a malaria pathogen such as *Plasmodium falciparum*.

18. A kit according to claim 17, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more malaria epitopes from the list given in table 1.

19. The kit according to claim 18, wherein the CD8+ T cell epitopes in (i) include all of the epitopes given in table 1.

20. The kit according to claim 16, for generating an immune response against HIV.

21. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more HIV epitopes from the list given in table 2.

22. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include all of the epitopes given in table 2.

23. The kit according to any one of claims 1 to 22, wherein the priming and boosting compositions are identical in that both contain the source of epitopes in (i) and the source of epitopes in (ii).

24. The kit according to any one of claims 1 to 23, wherein the priming composition and/or the boosting composition is in particulate form suitable for delivery by means of a gene gun.

25. A method for generating a protective CD8+ T cell immune response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to any one of claims 1 to 24.

26. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant non-replicating or replication-impaired pox virus encoding the same epitope or antigen.

27. The method according to claim 25, wherein the recombinant vaccinia virus is a recombinant MVA vector.

28. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen.

29. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against malaria such as *P. falciparum* malaria.

30. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against HIV.

31. The method according to any one of claims 25 to 30, wherein (ii) is delivered intravenously, intraepidermally or intradermally.

32. A medicament for boosting a primed CD8+ T cell response against at least one target antigen, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired recombinant poxvirus vector, and a pharmaceutically acceptable carrier.

33. The medicament according to claim 32, wherein the vector is a vaccinia virus vector such as MVA.

34. The medicament according to claim 32 or 33, for boosting a naturally primed CD8+ T cell response against malaria.

35. A method of boosting a primed CD8+ T cell immune response, which method comprises administering a medicament according to any one of claims 32 to 34.

36. The use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response.

37. The use of an MVA vector in the manufacture of a medicament for boosting a CD8+ T cell immune response.

38. An epitope string comprising the amino acid sequences listed in table 1.

39. A recombinant Ty-VLP comprising the epitope string according to claim 38, for immunising against malaria.

40. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the epitope string according to claim 38, for immunising against malaria.

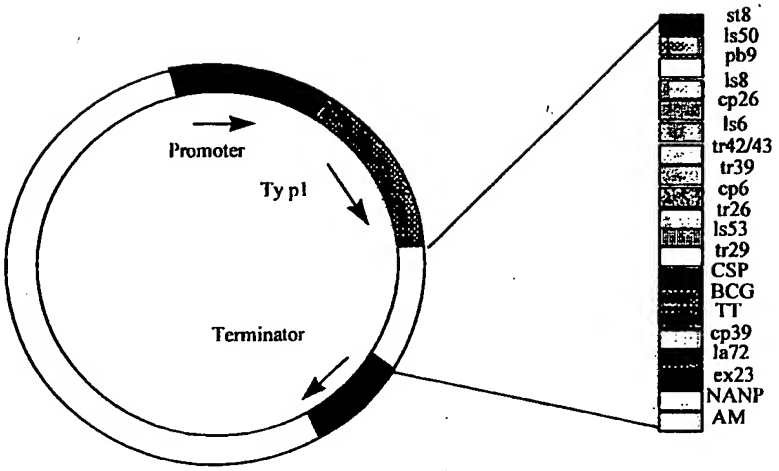
41. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the *P. falciparum* antigen TRAP, for immunising against malaria.

42. A recombinant vaccinia virus according to claim 40 or claim 41, of the MVA strain.

43. An epitope string comprising the amino acid sequences listed in table 2.

44. A recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes such as CTL epitopes from malaria.

Figure 1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Terminator
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pb9
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ls6
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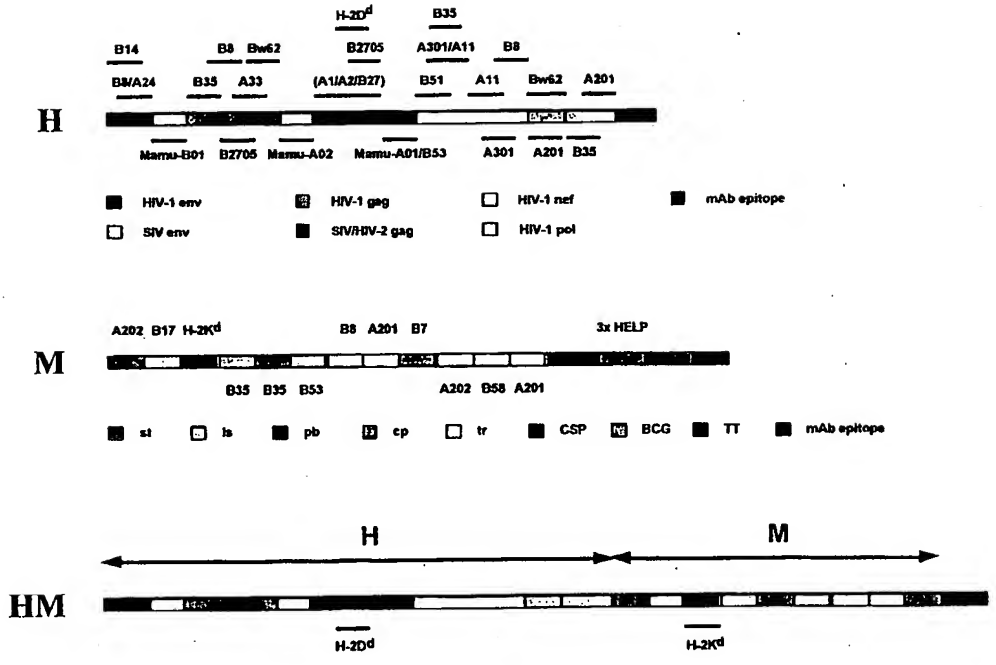
BCG
TT
cp39
la72
ex23
NANP
AM

Figure 2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A11
Bw62
A201
Mamu-B01
BZ705
Mamu-A02
Mamu-A01/B53
A301
A201
B35
HIV-1 env
HIV-1 gag
HIV-1 nef
mAb epitope
SIV env
SIV/HIV-2 gag
HIV-1 pol
A202 B17 H-2K^d
B8 A201 B7
3x HELP
B35 B35 B53
A202 B58 A201
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Figure 3

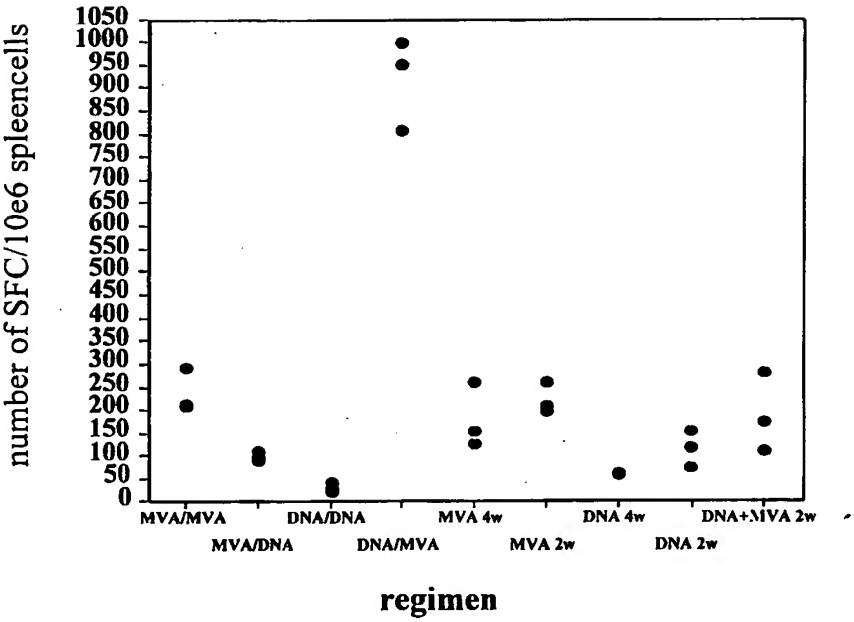
number of SFC/10e6 spleencells
1050
1000
950
900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
MVA/MVA
MVA/DNA
DNA/DNA
DNA/MVA
MVA 4w
MVA 2w
DNA 4w
DNA 2w
DNA+MVA 2w
regimen

Figure 4

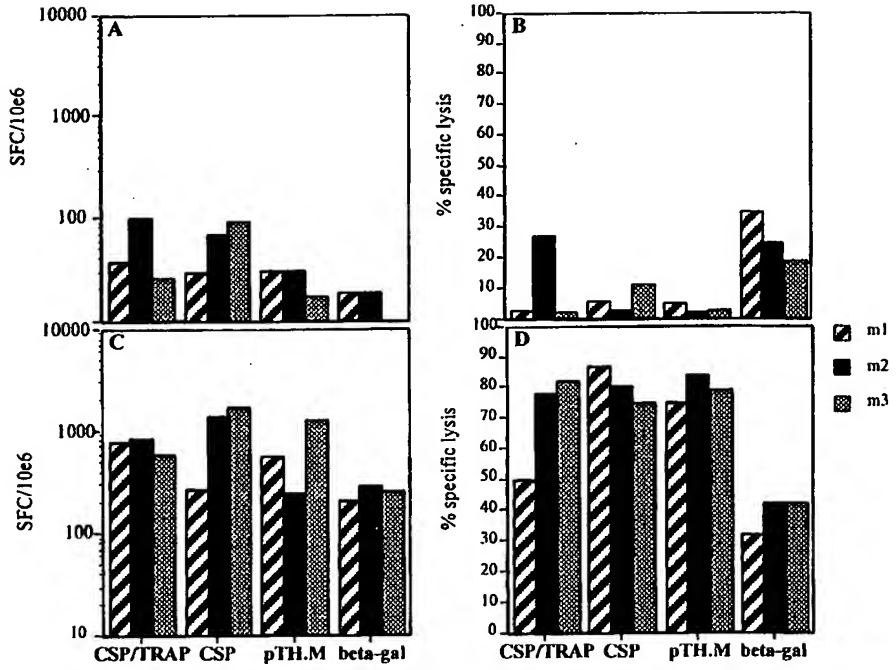
A
B
C
D
SFC/10e6
% specific lysis
10000
1000
100
10
90
80
70
60
50
40
30
20
0
CSP/TRAP
CSP
pTH.M
beta-gal
m1
m2
m3

Figure 5

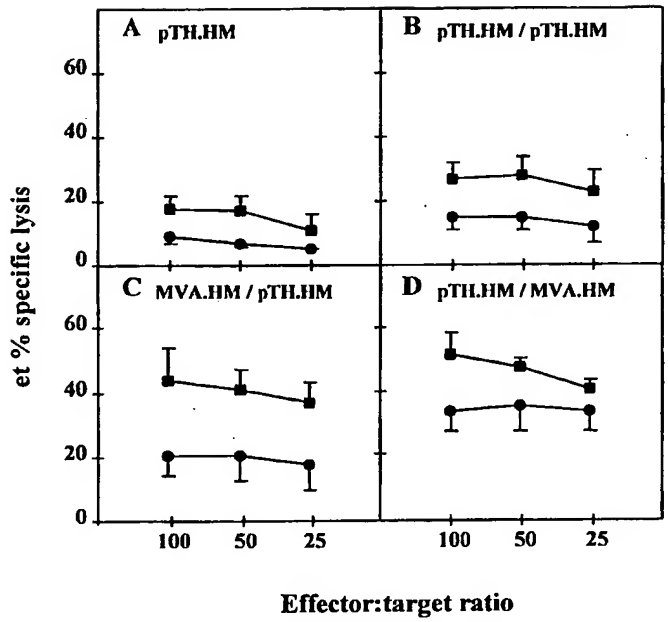

Figure 6

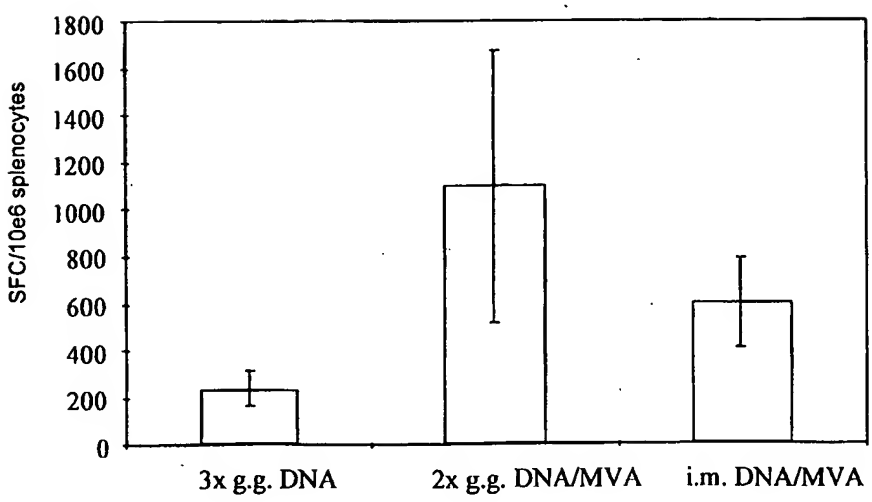

Figure 7

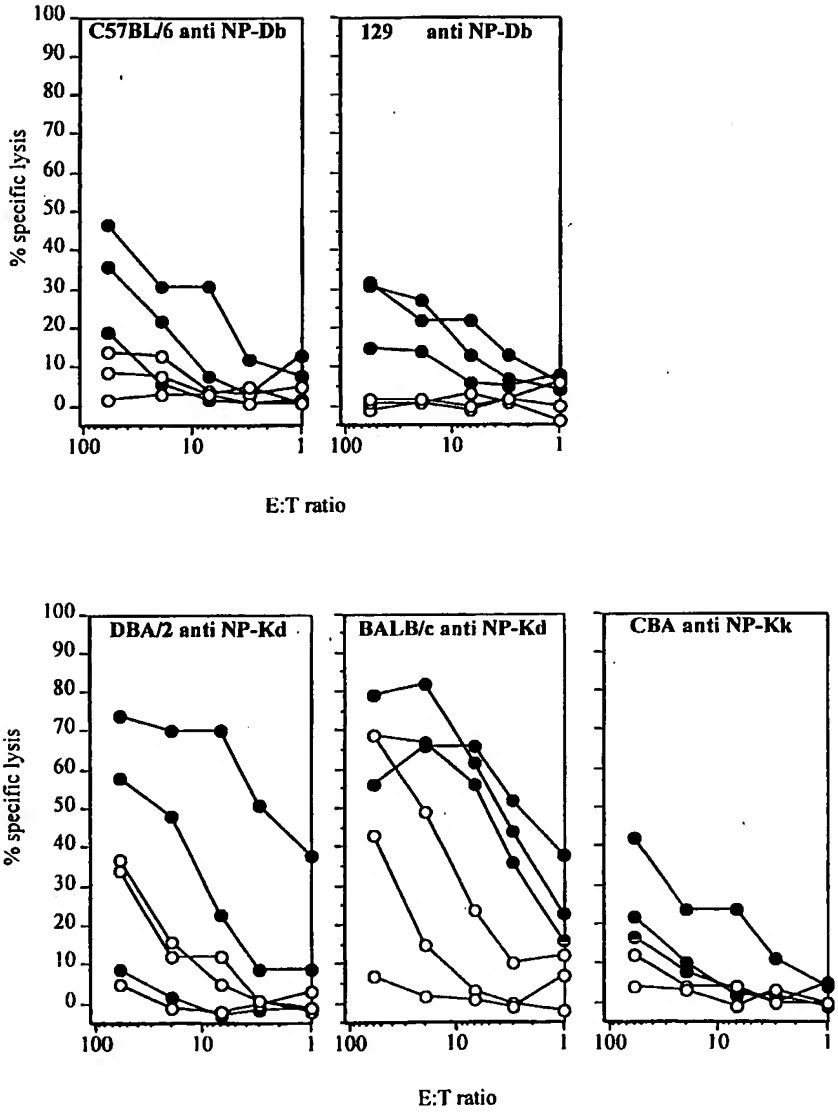
C57BL/6 anti NP-Db
129 anti NP-Db
% specific lysis
E:T ratio
DBA/2 anti NP-Kd
BALB/c anti NP-Kd
CBA anti NP-Kk
% specific lysis
E:T ratio

Figure 8

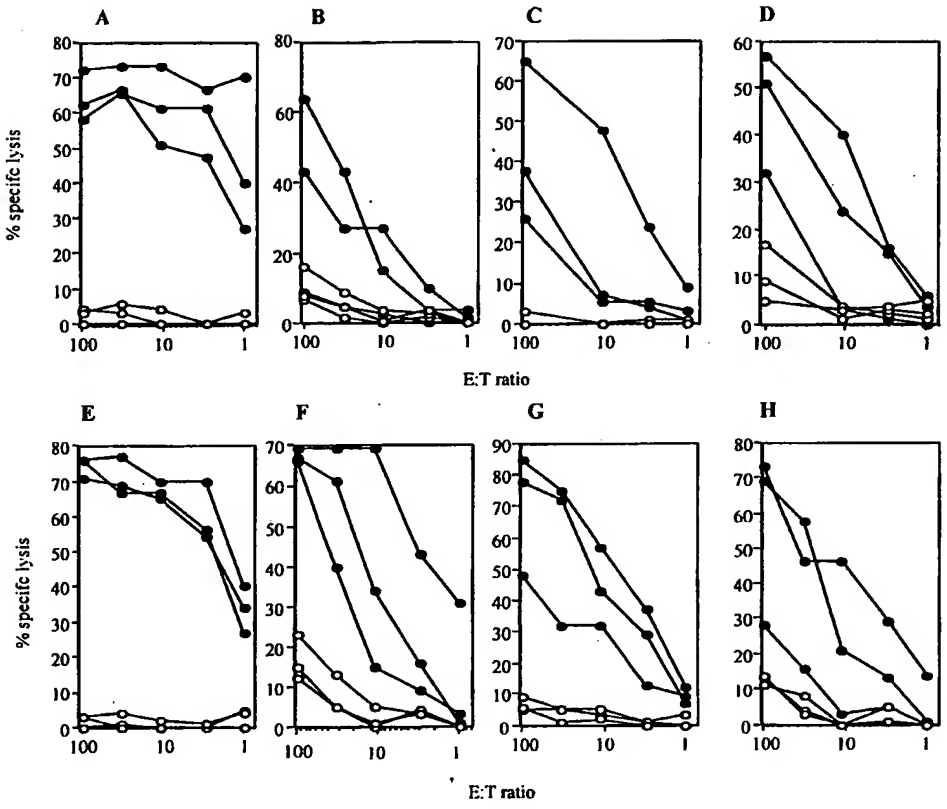
A
B
C
D
E
F
G
H
% specific lysis
% specific lysis
E:T ratio
E:T ratio
100
10
1

Figure 9

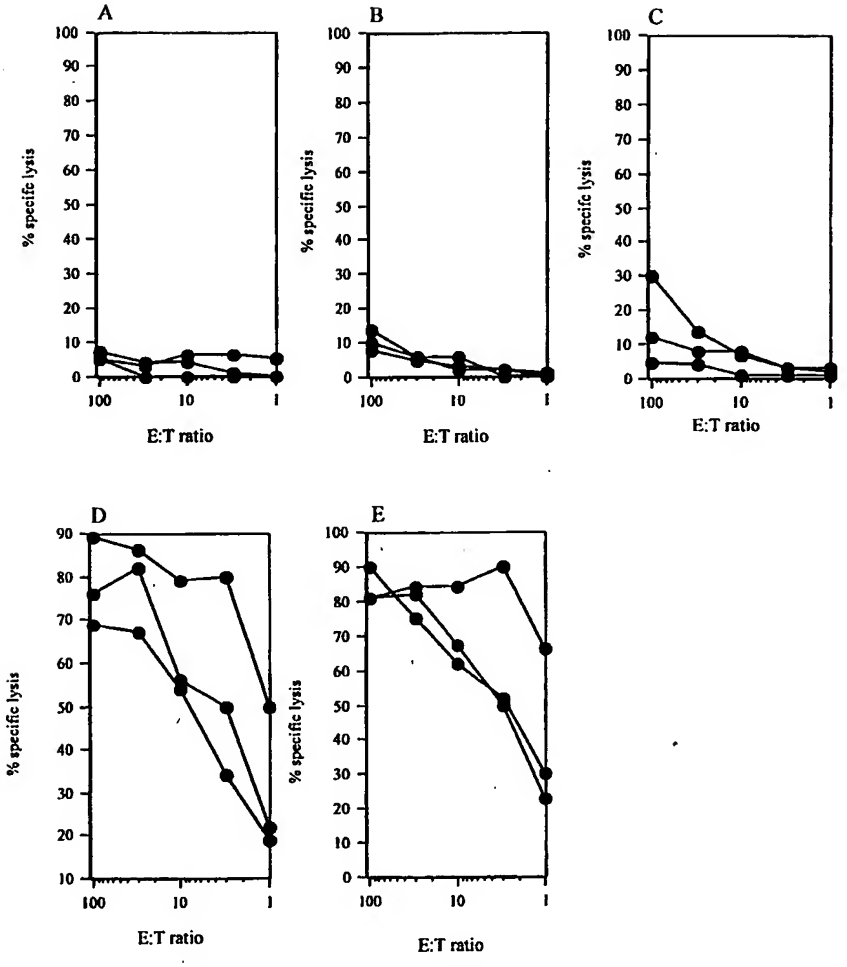

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Figure 10

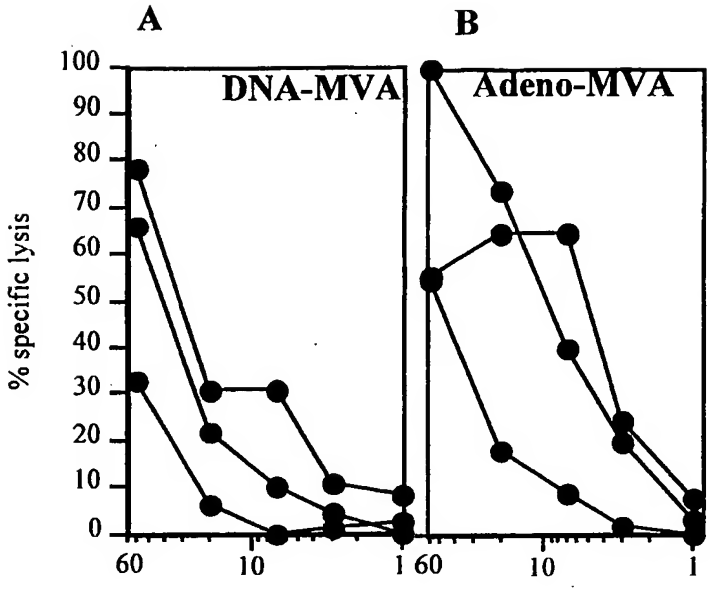

Figure 11

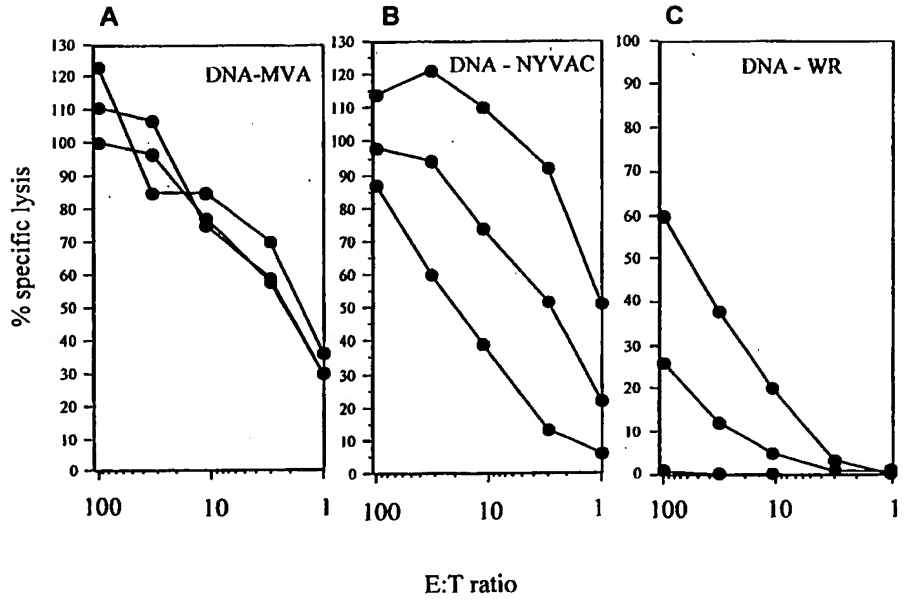

Figure 12

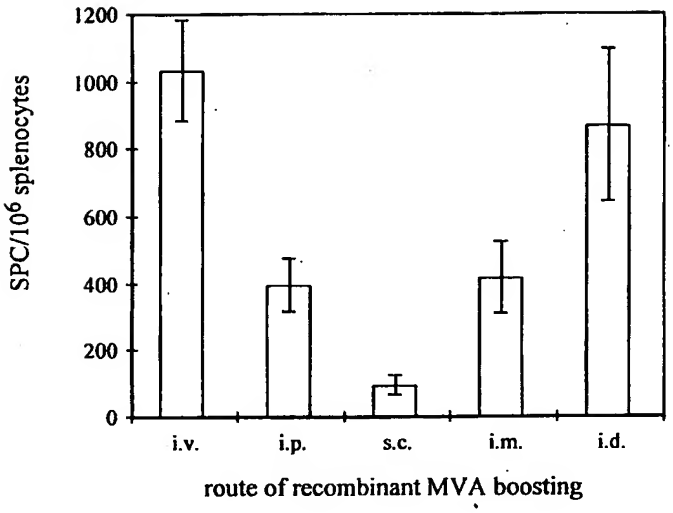

Figure 13

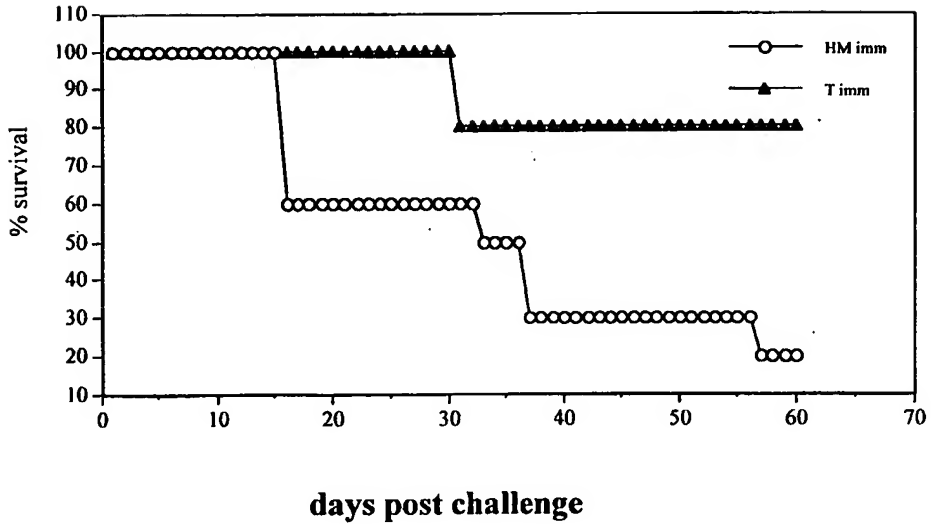

Figure 14

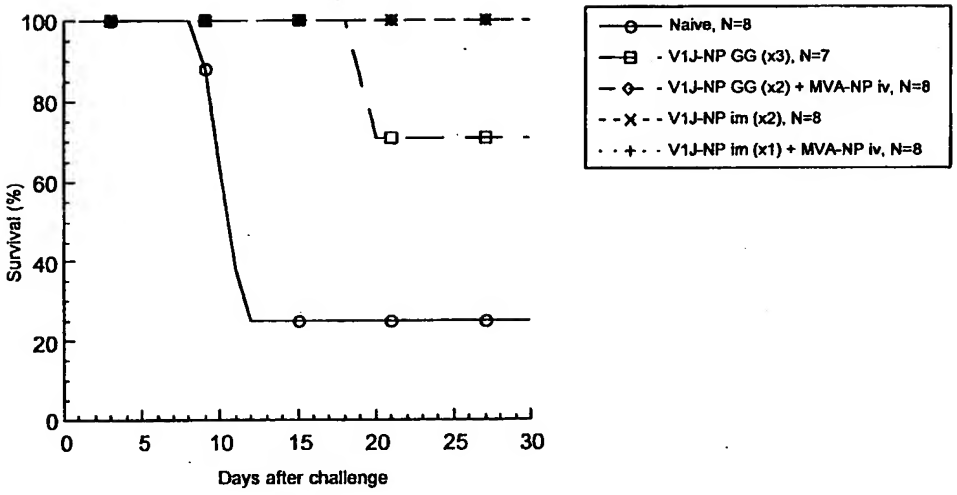
100
80
60
40
20
0
Survival (%)
0
5
10
15
20
25
30
Days after challenge
Naive, N=8
- V1J-NP GG (x3), N=7
- V1J-NP GG (x2) + MVA-NP iv, N=8
- V1J-NP im (x2), N=8
- V1J-NP im (x1) + MVA-NP iv, N=8

Figure 15

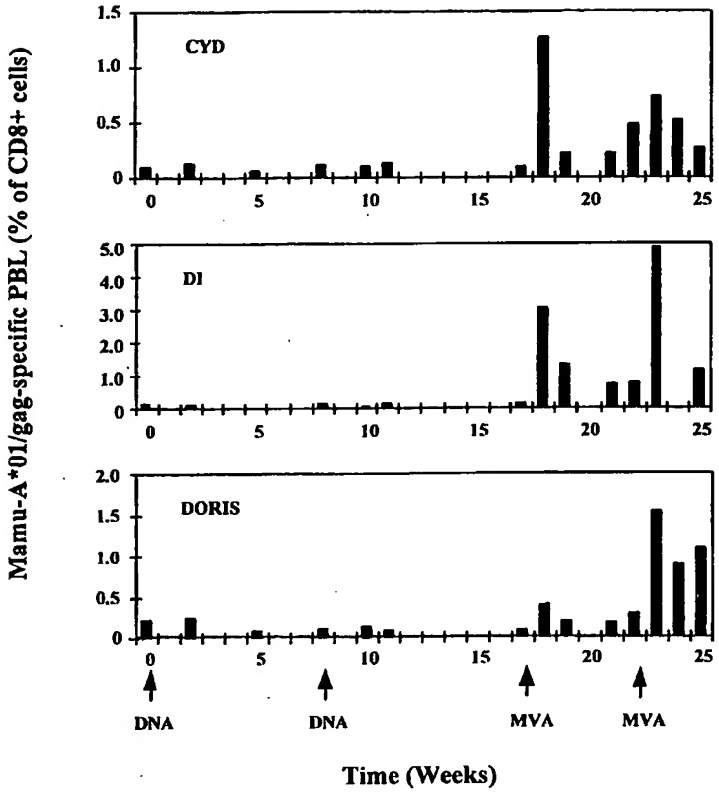

Figure 16